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What is wrong with US engineering?

To many of those outside the United States, the recent wave of anxiety about the quality of engineering in the United States, and the rate of industrial innovation in American industry, will seem curiously self-indulgent. How can it be, the question will be asked, that the industrial state which, since the turn of the century, has been the dominant source of new technology, and whose people now enjoy unprecedented and unparalleled prosperity largely as a result of past innovation, should so suddenly take fright? Is there something real to worry about? Or is it just another election stunt? What can be learned from experience elsewhere? And what, in any case, can be done?

The speed with which public concern has blown up is breathtaking, almost bewildering. The trigger appears to have been the recognition that the rate at which automobiles imported into the United States, especially those from Japan, may not be halted by the launching of the new indigenous products of the three principal automobile manufacturers, apparently more resolute than before to market vehicles that people are likely to want to buy as gasoline prices keep on increasing. The discovery, or rather the recognition, that the American car industry is less productive, man for man, than its Japanese competitor seems to have helped dramatize more problems of wider long-term significance — the weakness of the dollar, itself dependent on the continuing deficit of overseas trade and, to some extent, on the federal government's own domestic deficit.

In truth, this process of the discovery of crisis in the United States puts the cart before the horse. There have been signs of trouble for much longer than a decade. Since the early 1960s, and before the monetary rot set in, productivity (measured on a man by man basis) has been growing less quickly in the United States than in most other industrialized countries (the United Kingdom and, more recently, Italy excepted). Between 1955 and 1977, the annual growth of productivity in the United States has been an average of 2 per cent per year, only a third of the annual increase of productivity in Japan during the same period. More recently, there have been signs that the rate of investment in manufacturing industry has been declining (as a proportion of national wealth) as consumers have kept on spending, or providing funds for the federal government to spend on their behalf. Where, people are beginning to ask, will it all end?

The first need is not to take fright. There are some respects in which the relatively poor record of recent years is a consequence of earlier success. Thus the displacement of a large part of the labour force from manufacturing industry to the service industries, whose potential productivity is inherently less, implies that the rate of growth of productivity is likely to decline. In this respect, the rate of growth of productivity in the United States has been traded for the availability of health services, education and the like, which Americans have come to think of as essentials, which they would indeed prefer to see improved — and which they are well-heeled enough to afford. The concern in the past decade about environmental quality, and the regulations and restrictions which have followed, have similarly been bought with diminished growth of productivity. (This is not to say that environmental regulations should be swept away but merely that environmental quality is a public purchase; Dr Barry Commoner, the presidential candidate for the People's Party in November, is after all forever saying that "there is no such thing as a free lunch".) Success and prosperity spawn problems in other ways. It is necessarily harder to break new ground, as the United States has been doing for the past century, than to follow a path already trodden. In a world in

which international trade is acknowledged to be an important stabilizing influence, and in which attempts to bend the rules are relatively infrequent, it is inevitable that countries at the top, like the United States, will find their industrial supremacy in traditional fields from time to time eroded. Whole industries, footwear for example, have disappeared in the past; others (steel?) will do so in the future. If the automobile industry should find, next spring, that it cannot keep imports at bay, that will not mean that the world has come to an end but merely that the pattern of industrial production is being shared out internationally and economically.

That, at least, is what the textbooks say. The message will bring very little comfort to a Detroit in greater trouble even than at present. But there is a sense in which the pattern of industrial change cannot indefinitely be postponed, or can be forfended only at enormous and increasing cost. Several European countries have learned this lesson the hard way. British shipbuilding is merely the most obvious. In the United States, however, as elsewhere in the industrialized world, the record of the past few years has been confused by the consequences of the curious mixture of deflation and inflation ushered in by the first dramatic increases of the price of oil in the second half of 1973. By now there is ample evidence that, in circumstances like these, industrial investment grinds almost to a halt. The long-term consequences for productivity are plain enough. If worn-out and obsolete machinery is not replaced, and if new kinds of enterprises are not begun, then the growth of productivity must inevitably be diminished. No palliative exists. The only remedy is to avoid inflation and create circumstances in which industries (and people) respond rapidly to changing circumstances.

Last month's package of economic proposals from the White House is in this respect not especially relevant. To the extent that some expected tax revenue is to be forgone, people will have more to spend and some industries may begin to feel cheerful again. But the federal government's deficit and, possibly, the inflation rate, may be increased. By the time that Congress has finished with these proposals, the outcome may not be the stimulus to industrial recovery that President Carter is planning. More directly relevant, but again of dubious consequence, are the proposals that there should be more or less instant tax credits, paid in cash if need be, when manufacturers invest in new equipment. For what it is worth, a very similar proposal is a central part of the French budget for 1981, made public two weeks ago. The objective is laudable, but the results will not necessarily be those intended. If, for example, the chief beneficiaries are those manufacturers who are struggling to preserve industries whose economic foundations have long since disappeared, the cause of productivity will be hindered, not helped.

This is the background against which to judge the concern of the National Science Foundation with research in engineering, and with the training of engineers. The central issue is whether it will be possible, within the \$1,200 million budget of the foundation, to make a decisive change in the pace of industrial innovation. For the past several months, the foundation has been searching for a form of internal organization that will enable it to function more effectively in this respect (see *Nature*, 11 and 18 September). It also shares with other federal agencies ambitions to found cooperative research centres that will carry out research on behalf of groups of industries judged to be in need of technological innovation (and prepared to share the cost). The proposed reorganization looks like a leaf out of the book of the



UK Science Research Council, which has long since used its Engineering Board as an explicit means of fostering and improving engineering research in British universities. Even the admirers of the Engineering Board acknowledge that it has had an uphill struggle, however. The National Science Foundation may have better luck, given the stronger tradition of research (as distinct from external consultancy) among engineering faculties in the United States. But nobody should think that the cooperative research centres will have a decisive effect. Many will no doubt be useful, but the funds available do not bear comparison with what the mission agencies spend on development contracts.

By the same tests, the proposal that there should be a National Engineering Foundation, with terms of reference similar to those of the National Science Foundation, is likely to fly like a lead balloon. Representative George Brown, the chief sponsor of the scheme, sees it chiefly as a way of goading existing institutions into action. But what action? As long as nobody is sure, a National Engineering Foundation would be simply a cosmetic device, a monument to the common Washington view that any problem can be solved by throwing money at it.

Yet there are constructive things that could be done. In the past twenty years, the character of engineering has changed

enormously (especially in the United States), but the engineering schools remain much as they were. The best of them are prestigious places, and have adapted their teaching to changing needs. Elsewhere, teaching and the evolution of the curriculum have been hampered as much by the lack of teachers as money. If the eager beavers in Washington really seek to help, they would be well advised to concentrate on the education of engineers.

The consequences (so far) of the Finniston Inquiry into British engineering education may have been meagre, but the more flexible American university system could well benefit from taking stock of how it educates engineers. Why, for example, should not some engineering schools follow most medical schools in recruiting intending engineers from among people with good first degrees in relevant fields of study? These and other innovations deserve a fair hearing. Some useful ideas are, by all accounts, included in the report of the Secretary of Education and the Acting Director of the National Science Foundation, submitted two months ago but not yet published. Congressmen urging that something should be done about engineering might do worse than demand a sight of that document. But in the long run they must know that the health of the engineering profession depends intimately on the health of the economy, which is Congress's chief responsibility.

Poor outlook for British universities

One thing is certain about the future of the British university system — if it collapses, or if some parts of it collapse, these events will be recorded in the appropriate Annual Survey of the University Grants Committee (UGC) with decorum and gentility. These are the hallmarks of the latest document in this series, published last week (HMSO, Cmnd 8031) and covering the turbulent academic year 1978–79. The UGC records the successive chops and changes of public support for the universities under its wing, and does not in even the mildest language protest that this is not the way in which a motley collection of universities such as the British can be run sensibly, even economically. In the same way, the latest survey tells how the UGC responded to the decision a year ago that the recurrent grant to the universities would be slightly reduced in the academic year now starting by “advising universities . . . that their admission arrangements for 1980 should be so conducted that, if necessary, they could restrict the number of entrants in a way which would restrict the rate of increase in the total numbers of home undergraduates”. The survey notes that the UGC “subsequently learned” of the proposals to increase the fees of students from overseas and says that “the unpredictable results of these new arrangements are causing universities much anxiety”. The UGC as such stays on the fence, not adding even a mild expression of opinion such as “which we share”. They order things differently in France (see page 381).

The truth is that the upshot of government decisions about British university finances in the past two years amounts to the most serious perturbation of the system since the onset of rapid expansion in the early 1960s. The bursars' departments of British universities have become almost as concerned to know what cheques will be in the next post as the most insecure small businesses. The comfortable quinquennial system of financing will not be revived in the foreseeable future, but even the system of rolling budgets offered as a substitute four years ago is vulnerable, from day to day, to quite minor changes of government policy. The UGC itself, in the course of distributing funds to the universities in its charge, frequently finds it necessary to keep back funds for dealing with unexpected problems (a sudden pay award, perhaps). The result is that individual universities sometimes do not learn until towards the end of an academic year whether they will finish up in the black or the red.

The coming academic year will be worse than ever, for the rules have been changed. The UGC's own recurrent grant has been reduced by the estimated cost of educating new students from

overseas. Individual universities hope to make good the difference by the increased fees they will recover. It is too soon to know how successful they will be — not all the overseas students offered places have turned up as yet, while it remains to be seen how many will actually have the cash required of them. Some universities, a little surprisingly, seem confident that they will not be much worse off than last year. Others are despondent. It is, however, agreed that total income will decline. The UGC's own reaction is familiar, and understandable. Again it will keep some money up its sleeve, with the intention of helping out universities in trouble. As so often in the past, the successful universities will no doubt be penalized to help those that cannot recruit their expected quota of students from overseas. In the meantime, many university finance officers will be literally unable to make their budgets balance for the coming year. So they will skimp wherever they can, even on the quality of teaching.

Few outside the university system appreciate the debilitating consequences of this way of carrying on. The UGC no doubt regrets the way it has to keep its supposedly autonomous universities on tight and unpredictable purse-strings — but there is not a flicker of regret or even sympathy in the latest annual survey. This omission, tactful though it may be, is important and potentially damaging because there are already many in the universities who question the utility of the long-term survival of the UGC. For several decades, the committee has been held up as a shining example of public administration, a buffer between the government (first the Treasury, now the Department of Education and Science) and the universities. There may be no truth in the suspicion that the UGC is now less staunch a buffer, but its ready compliance with each tiny shift of public policy has given a different impression. That is merely one reason why it should seize what chances come its way to say where it stands. Another is that in the upheavals that lie ahead, some at least among British universities will look to the UGC for protection, not just from penury but from the structural changes there will be if the research councils eventually decide to concentrate their research grants on a smaller number of institutions (as the Merrison committee may well suggest). If the UGC persists in its present passive ways, however, it will be less able to offer help than the circumstances require. The larger and more successful universities will be able to look after themselves — and some would prefer to do so even as things are. There is no unalterable law that all universities must indefinitely survive. The UGC, however, seems well set to let them go without a struggle.

NASA budget falls foul of contracts

Washington

The National Aeronautics and Space Administration (NASA) has been caught in crossfire between the US Congress and the Office of Management and Budget (OMB) over whether the federal bureaucracy is spending too much money in employing private consultants to carry out its work. This has resulted in a demand from the Senate that NASA cut \$14 million from a budget for consultant services which NASA claims totals only \$4.3 million, but which the Senate Appropriations Committee, using a broader definition disputed by both the agency and the OMB, claims to be more than \$90 million.

Unless it is reversed during negotiations with the House, the extra cut in NASA's budget, coming on top of a proposed two per cent reduction (approximately \$100 million) in its budget request, will mean further pressure on space research projects as efforts are made to protect the space shuttle programme.

The pressures are already beginning to bite. For example, NASA is planning a reduction in the scope of experiments to be carried out by the spacecraft which will take part in the international solar polar mission, due for launch in 1982. Scientists from the European Space Agency, which is providing the second spacecraft in a dual mission, argue that the omission of instrumentation from the US craft could reduce the value of their own findings.

The proposed cuts in NASA's consultancy contracts reflect a general unease in Congress about the way in which federal agencies have come to rely increasingly on outside contractors to meet their responsibilities.

However, the agencies face a dilemma. On the one hand, Congress is continually increasing their work-load while, on the other, the Administration is keen to keep down the cost and size of government. The inevitable result is that the agencies contract work out. This, in turn, provides ample scope for disputes over rigged bidding for contracts, favouritism towards contractors, duplication of work and conflicts of interest.

Several instances of such practices have come to light in the past year. Last week, for example, officials from the Department of Energy admitted before a congressional oversight hearing that they had failed to check up on a consultancy firm employed to help draw up clean air regulations and were therefore unaware that the firm had also been working for companies engaged in a campaign to oppose the Clean Air Act of 1977.

In debating the 1981 budgets for a number of independent federal agencies, the Senate agreed to a proposal from Senator Warren Magnusson, chairman of the Appropriations Committee, to make a

15 per cent cut in the consultancy budgets of three agencies — NASA, the Environmental Protection Agency and the Department of Housing and Urban Development.

In opposing the proposed cuts, NASA complained that the analysis on which they had been calculated did not reflect the difference between narrowly defined contracts for advisory services and service contracts essential to a range of space programmes, including the shuttle.

Strong support for NASA's activities came from Senator Adlai Stevenson and ex-astronaut Senator Jack Schmitt, chairman and ranking minority member of the Senate Science and Space Subcommittee. Mr Schmitt proposed as an alternative that the 15 per cent reduction be according to NASA's own definition of what constitutes a consultant — this would reduce its budget by only \$640,000.

However, Mr Magnusson and fellow Appropriations Committee member Senator William Proxmire remained firm. Mr Magnusson quoted a recent report from the General Accounting Office citing OMB's apparent failure significantly to reduce excessive waste, and its abuse of consultants' contracts over a 20-year period, as well as a statement from Admiral Hyman Rickover that "the use of consultants often impedes, rather than facilitates, action by government agencies". Mr Schmitt's amendment was

lost by 27 votes to 66.

NASA is not the only agency in trouble about consultants. The Environmental Protection Agency (EPA) announced last Friday the cancellation of a \$285,000 contract offered to a research worker at the California Institute of Technology to study the health effects of radon gas because of charges that the contract had improperly been awarded to the university without competitive bidding.

Dr David M. Rosenbaum, head of EPA's Office of Radiation Programs, had offered the contract to the university without seeking other bids on the basis that Caltech was the only contractor qualified to carry out the work involved. However, a university research worker told a Senate committee last Thursday that there were other contractors who could have carried out the same work.

Dr Rosenbaum also justified his actions on the grounds that it would have taken nine months to secure the services of a consultant through competitive bidding, and that because radon was a "serious health problem", fast action was needed.

However, his superior at EPA said that Dr Rosenbaum may have violated the agency's procurement regulations in making the award. And the agency's inspector general said that she had found "serious and troublesome" problems in the contracts procedures used by the radiation office.

David Dickson

Einstein Observatory in trouble

Washington

Equipment problems on board the National Aeronautics and Space Administration (NASA)'s second High-Energy Astronomical Observatory (HEAO 2) — also known as the Einstein Observatory — are causing concern that the mission may have to be brought to a premature end.

Initially the satellite, which was launched in 1978 and has provided the first X-ray telescope as sensitive as ground-based optical telescopes, was planned to operate only for one year. But after its initial success in generating new scientific data, the mission was extended, and until the recent setback NASA scientists hope the satellite would continue to send back data well into next year, when atmospheric drag would take it out of orbit.

How long the satellite goes on operating, however, will now depend on the behaviour of the gyroscopes used to position it. Three weeks ago, the transmission of scientific data had to be temporarily shut down after the failure of two of the six gyroscopes to switch on after a temporary black-out.

The satellite needs three functioning gyroscopes to position itself. One is already

dead, and another has been working erratically for some time. The latter, however, has now had to be brought back into service in the hope that its performance will be adequate. If not, NASA officials said last week that they are developing software instructions for a back-up control system which would use the two gyroscopes that are still functioning, as well as either a sun sensor or a star tracker on the satellite.

At Harvard University, whose Center for Astrophysics is responsible for collecting the data transmitted from the Einstein Observatory, scientists also fear that even if the observatory continues to operate satisfactorily — as they are now hoping — the extra fuel consumed during recent manoeuvring to keep solar power cells facing the sun will shorten its lifetime by a couple of months.

The trouble on HEAO 2 started when one of the thrusters on the satellite started to burn for longer than it should during a repositioning manoeuvre. The gyroscopes were subsequently turned off with other equipment, but two failed to start when power was switched back on.

The gyroscopes have given trouble once before at the start of the mission in 1978,

but this was resolved by NASA engineers. Ironically, while the problems with HEAO 2 were being worked on, gyroscope problems also developed in its successor HEAO 3, which was intended merely for a six-month mission.

In this case, a command sent to HEAO 3 caused it inadvertently to start drifting from its proper position, leading its control computers to switch the satellite into a "safe" mode. However, this problem is again said by NASA engineers to have been solved.

The HEAO satellites are not the only space activities encountering technical problems. Thus NASA officials are now carrying out an in-depth review of the space telescope programme and are concerned about the possible combined effects of cost overruns and schedule slips.

The critical design review of the telescope has been put back from this summer until January 1981, partly because of the need to redesign the wide-field planetary camera to save weight. In addition, the fine-polishing of the primary mirrors for the telescope is behind schedule; and various contractors for the space telescope project, due to be launched from the space shuttle in early 1984, are complaining that they have been allocated insufficient manpower and funds to keep the programme on schedule.

NASA is soon expected to announce the results of a competition for the location of the Space Telescope Institute, which will collect and analyse data from the telescope. The three main contenders are Johns Hopkins University in Baltimore, Princeton University and the Fermi National Accelerator near Chicago. Two groups — Associated Universities Inc. and Universities Space Research Association — have drawn up separate plans for locating the institute at Princeton. The Baltimore site is being proposed by the Association of Universities for Research in Astronomy and Fermilab by the Universities Research Association Inc., which already operates the laboratory's accelerator for the Department of Energy.

David Dickson

Radiation safety

X-ray survey

A plea for more effective steps to reduce the radiological dose to the gonads of patients from X-ray diagnosis has been put out by the National Radiological Protection Board (NRPB). The board is especially concerned about young adults, and says that if they were protected as well as children usually are, then the genetically significant dose to the British population from diagnostic radiology could be reduced by 40–50 per cent.

The NRPB set out to investigate whether the lessons from a similar study in 1957 under Lord Adrian had been learnt. That study concluded that the dose to the gonads

of individuals could be reduced by the use of better radiological techniques, such as narrower beam widths. It also found that gonad dose from the same types of examination varied considerably, sometimes by as much as a factor of three or four between different parts of the United Kingdom.

The new study has found that the genetically significant dose — the average gonad dose per head of population weighted for child expectancy data — has remained approximately the same over the past 20 years despite a 50 per cent increase in the annual number of radiological examinations, suggesting that techniques have indeed improved. But it has also found that local variations in dose for the same examinations are as great as they were in 1957, suggesting that the use of gonad protection varies from place to place.

With this said, the NRPB is not alarmed. The "genetically significant dose" in Britain is still considerably less than in most other industrialized countries and could be responsible for a total of 100 genetic defects a year compared with a rate of 20,000 cases of genetic birth damage each year. Nevertheless, according to one of the reports, a reduction of only 10 per cent in the contribution to the genetically significant dose from diagnostic radiology would be the equivalent of the present contribution from nuclear power. As techniques for reducing the radiological contribution are easily available and relatively inexpensive compared with those needed to achieve a similar reduction from nuclear power, the NRPB says they should be implemented.

The study, based on data about radiological investigations in 1977, measured the gonadal dose in a sample of patients undergoing different types of examinations. The genetically significant dose is inferred from child expectancy data.

A breakdown of examinations into type reveals that most have increased in frequency whereas a few have decreased. A substantial factor in keeping the genetically significant dose down to its 1957 level is the large reduction in the number of obstetric radiological examinations. Their contribution to the genetically significant dose has fallen from 4.5 mrad in 1957 to 0.6 mrad now.

Although the NRPB study claims that there is probably room for further reductions of the genetically significant dose, it has few practical suggestions as to how this might be done. Nor does the NRPB fully understand the large differences of gonadal doses for the same examination in different parts of the country. It also acknowledges that gonad shields cannot be used in all cases, especially where they would obscure organs to be investigated. The plan is to discuss the findings with radiologists later in the year in the hope of finding ways to reduce the genetically significant dose.

Judy Redfearn

DNA guidelines

More relaxation

Washington

The National Institutes of Health (NIH) are expected shortly to reduce the burden of recombinant DNA regulations on research workers. This follows the recommendation of the institute's Recombinant DNA Advisory Committee (RAC) that details of virtually all experiments covered by the current safety guidelines need no longer be submitted to NIH for review.

At the same time, RAC has decided that NIH should limit their attempts to oversee recombinant DNA activities carried out in the private sector. In particular, the committee has proposed a procedure for checking on the biological aspects of large-scale experiments, but is suggesting that it should now restrict itself to general comments on the physical aspects of the fermentation and containment technology rather than reviewing each proposal submitted.

RAC has not gone as far as some would like. At a meeting in Bethesda, Maryland, last week, it decided to defer action on a proposal that clearance from local institutional biosafety committees (IBCs) should no longer be required before a research worker carries out an experiment in containment conditions specified in the guidelines.

The proposals for a procedural change in the guidelines were put to RAC by Dr Maxine Singer, head of the National Cancer Institute's biochemistry laboratory and a prominent participant in the 1973 Gordon conference which first drew attention to the need for caution in recombinant DNA research.

Dr Singer told the committee that many scientists felt that there was unnecessary delay in waiting for the Memoranda of Understanding and Agreement, required by NIH, to be approved by IBCs before research was allowed to begin. Also, there was impatience with the requirement for central review of experiments where the safety procedures to be followed were relatively straightforward.

On the latter point, committee members readily accepted Dr Singer's proposal that central registration be eliminated. Such registration is already no longer required for the bulk of recombinant DNA experiments, those carried out with the disabled K12 strain of the bacterium *Escherichia coli*. The new proposal would chiefly affect experiments being conducted in P2 and P3 physical containment conditions; the status of experiments now requiring the NIH director's explicit approval would not be altered.

More controversial was the proposal to remove the requirement for prior review of experiments by IBCs. Debate on this was coloured by a report presented to the committee on the performance of 19 IBCs

studied in the state of California. This revealed, among other things, considerable differences in the rate at which IBCs accepted or rejected proposals for experiments in particular containment conditions, raising questions about the consistency with which research workers are interpreting the guidelines.

The report was prepared by Dr Dianna Dutton of Stanford University School of Medicine, under a grant from the National Science Foundation. She pointed out that IBCs established by private companies in particular had a high acceptance rate of proposals, and also claimed that the fact that none of the IBCs contacted publicized their regular meetings, even though attendance was unrestricted, reflected an apparent reluctance to involve the public in IBC decision-making.

A nationwide study of IBCs is now being planned by NIH, and will be discussed at a meeting of IBC chairmen in November. In the light of this proposed review, and the result of the Stanford study, RAC agreed that consideration of the elimination of pre-review of experiments should be deferred "until the frequency of principal investigator error in selecting containment levels is determined".

In another step designed to lessen the burden of regulation — this time primarily on the private sector — the committee agreed that its review of proposals for large-scale experiments should be focused chiefly on aspects of biological safety.

This decision was the culmination of a lengthy debate over how far NIH should go in regulating recombinant DNA research in the private sector. As there is no legislation, private companies are at present unregulated, although all companies involved have agreed to observe the NIH guidelines voluntarily.

A major stumbling block to this has been disagreement over procedures for evaluating experiments involving more than 10 litres of culture, at present requiring special permission from the director of NIH regardless of the genetic material being used. Some committee members have argued that RAC lacks the technical expertise to evaluate the safety of large-scale fermentation techniques, others that regulation in this area is the responsibility of the Department of Labor's Occupational Safety and Health Administration (OSHA).

Dr Eula Bingham, however, the head of OSHA, has now written to NIH saying that although her agency can act on complaints from workers or on suspicion of a potential hazard, it lacks the statutory powers to require the certification of facilities where no hazard is suspected.

Committee member Dr Sheldon Krimsky of Tufts University proposed that, in the light of OSHA's reply, a subcommittee of RAC should be set up with representatives of other federal agencies to comment on proposed containment procedures for large-scale

experiments. He pointed out that although the pre-review of industrial technology might be a radical departure for regulatory agencies in general, it was within the spirit of the scientific and public concern which had originally led to the development of NIH guidelines.

Other committee members, however, were not convinced. Having earlier passed a motion agreeing to procedures for evaluating the biological classification of proposed large-scale experiments — and therefore the level of physical containment that would be required — they amended Dr Krimsky's proposal suggesting a new subcommittee primarily to advise on the proper procedures and design of such operations. The proposed subcommittee would review the effectiveness of local IBCs, including industrial IBCs, but it would not have to approve specific containment procedures for particular experiments; and its creation has still to be approved by the director of NIH.

David Dickson

Biotechnology

UK company gells

Britain's new biotechnology company, Celltech, is beginning to take shape. Sir Michael Stoker, foreign secretary of the Royal Society and former director of the Imperial Cancer Research Fund, has been appointed chairman of the board of directors and it is thought that Norman Carey of G. D. Searle Limited is in line for the scientific directorship. Most of the other members of the board have also been appointed. Offers of senior posts have been made, although it is not yet clear how many of them will be taken up. A full statement is promised later in the month.

Since the National Enterprise Board's announcement in July (*Nature*, 24 July, page 321) that it was to provide 40–50 per cent of the initial £12 million to set up the company, the managing director, Mr G. Fairtlough, has been looking for a site for administrative offices and a laboratory as well as appointing his board and senior posts. The most likely site, as was rumoured earlier, is Cambridge, although other possible sites are also being considered. The plan now is to buy existing offices and laboratories near to each other, which could be adapted easily to the company's needs.

The timing of the start of the company's operations will depend to a large extent on the suitability of the laboratories it acquires, but Mr Fairtlough is hopeful that operations could begin early next year. An older building needing extensive alterations would obviously delay the start.

As soon as the senior post have been filled, Fairtlough plans to start looking for candidates for other posts. The company hopes to build up a staff of 75–100 over the next two years. Initial products will be for medical diagnosis, but the company hopes

Going and coming

Sir William Henderson, chairman of the Genetic Manipulation Advisory Group (GMAG), responsible for the administration of the UK guidelines on recombinant DNA, will be giving up as chairman at, or soon after, the end of 1980. His original appointment, at the end of 1978, was for a term of two years, but it is understood that he is prepared to stay on for a month or so if his successor cannot take over at the beginning of the year.

In the past few months, there has been some confusion among GMAG members about the future chairmanship of the committee, which has apparently not yet been decided.

GMAG is also in the throes of a debate about its own future. At its next meeting, GMAG will debate a proposal by one of its members that the group should recommend its own demise. The proposal is likely to be resisted by the government departments and agencies concerned, at least until the Dangerous Pathogens Advisory Group (in limbo since the smallpox accident at the University of Birmingham two years ago) is reconstituted.

It has also become known in the past week that the search committee of the European Molecular Biology Organization (EMBO) has nominated as the next director-general of EMBO Dr L. Philipson of the Department of Microbiology of the Biomedical Centre at Uppsala. If appointed by the EMBO council, Dr Philipson will succeed Sir John Kendrew, whose term of office ends next year.

to expand into other fields later and to cooperate with larger pharmaceutical companies on production of items beyond its scope.

Judy Redfearn

French universities

Trouble behind

Paris

French universities are still recovering from a series of attacks and retreats by the government which have left the universities strictly on the defensive.

In July, the minister for the universities, Mme Saunier-Seïté, vetoed the granting by the universities of the long-standing "engineer PhD", the Docteur-Ingénieur, and cancelled 200 first degree (deuxième cycle) and 600 postgraduate (troisième cycle) courses which were due for reapproval by her ministry.

Last month, the Prime Minister, M. Raymond Barre, intervened in the resulting row, but his action was more cosmetic than real. He restored the freedom to create Docteur-Ingénieurs at 25 of France's 78 universities, and approved a further 22

courses at first degree and 39 at postgraduate level, after energetic lobbying by the Conference des Présidents d'Université during the summer.

According to the vice-president of the Conference, Professor Jacques Latrille (president of the Université de Bordeaux II), these concessions are "nothing like enough". Last year some 2,000 Docteur-Ingénieurs qualified and 25 universities cannot cope with the demand, which comes largely from qualified students of the Grandes Ecoles wishing to do research. Although the Grandes Ecoles, sometimes described as the crack training grounds of the French establishment, have a highly selective entry, they offer relatively few opportunities for research.

The scrapping of a net 178 first degree courses will — by government design — chiefly affect the new universities, the score of institutions, such as the universities of Chambéry and Pau, which have been established in the French provinces since the reorganization of 1968 and which tend to have 3,000–5,000 students. The social sciences, educational science, psychology, philosophy, history, geography, foreign languages and the performing arts have all been hit hard, with the result that teaching in these disciplines will be concentrated in the major cities and Paris. There may be further cuts next year, when the other half of the 1,917 undergraduate diplomas will be up for renewal.

The postgraduate cuts, on the other hand, affect mainly the bigger, older universities (15,000 to 20,000 students each), where most research is done; but again the cuts fall mostly in the humanities.

M. Barre, in his recent speech, gave his support to the broad emphasis of Mme Saunier-Seïte's policy — the restoration of "the vigour and quality" of the universities after the "rude shock" of 1968. It was clear, said M. Barre, that an increasing number of postgraduate (troisième cycle) proposals were ill thought-out and sustained by too few professors, libraries and laboratories.

A survey of deuxième and troisième cycle courses by Mme Saunier-Seïte had revealed a single management professor requesting the establishment of three postgraduate courses; six professors of belles lettres proposing ten senior undergraduate courses; and five biology professors proposing three postgraduate courses in life sciences.

M. Barre mentioned the notion of autonomy, but it is unlikely that the state will easily relinquish its vast power to approve all courses as well as appointment of professors and lecturers. On this point, the Prime Minister contrived to sound more like a benevolent dictator, offering democracy to his people "when they were ready", than a man committed to granting independence.

Neither does Mme Saunier-Seïte offer many crumbs of consolation. Although she is president of the Conference des

Présidents d'Université, she has attended none of its meetings for more than a year. Academics are not asking for revolution. The government, says Professor Latrille, is entitled to make its own policy for the universities: but the universities should at least be consulted, through existing mechanisms, to give advice on how it is applied.

Robert Walgate

Non-proliferation

India gets fuel

Washington

Last week was a good one for the US nuclear industry, and a disappointment for its critics. On Tuesday, voters in the state of Maine rejected a proposed law which would have banned nuclear power from the state. The next day the US Senate failed to prevent the export of nuclear fuel to the Tarapur nuclear power station in India despite India's continued refusal to submit its nuclear facilities to international inspection.

Both votes had been closely watched as pointers to nuclear policy in the 1980s and both had, in consequence, been the focus of intense lobbying. In the lobbying in Maine, for example, supporters of a bill which would have closed the state's single nuclear power station and forbidden the construction of further nuclear plants were outspent by five to one by pro-nuclear forces, who raised financial support from utility companies around the country.

Although the vote in Maine supports the development of nuclear power, anti-nuclear voters did turn out in strength, and 40 per cent of those voting supported the proposed ban. After the vote, a spokesman for the local utility company admitted that the result demonstrated the substantial public concern that exists about the safety of nuclear power.

In the Tarapur case, the Administration's victory was even closer, and the lobbying even more intense. Shaken by a defeat by three to one in the House of Representatives of its attempts to permit the export of the nuclear fuel and a subsequent defeat in the Senate Foreign Relations Committee, the Carter Administration pulled out all the stops to prevent what was seen as a major challenge to its foreign policy.

Many key senators received telephone calls from the President, and Secretary of State Edmund Muskie even pleaded at length with the Senate Republican Conference. The result was that a resolution to disapprove the export of the fuel was defeated by just two votes, 46 to 48, with a majority of Democrats voting against the resolution and the majority of Republicans — with a significant number of liberal Democrats — voting for it.

India's request for the export of 38 tons of fuel for its Tarapur reactor was the first major test of the Nuclear Non-

Proliferation Act of 1978, which forbids the export of nuclear fuel to countries which have not accepted international safeguards on their nuclear facilities.

Earlier this year, the Nuclear Regulatory Commission (NRC) decided unanimously that, since India had not met these requirements, the sale of the fuel could not go ahead. President Carter, however, invoked broader foreign policy considerations to overrule the NRC's decision, and the President's move could only have been overturned by Congress if both houses had voted against it.

The dispute over the export licence resulted from a conflict of strategy about whether non-proliferation objectives should be pursued single-mindedly, as implied by the Non-Proliferation Act, or whether they are as likely to be achieved through more conventional economic and diplomatic channels.

Senator John Glenn, the leading supporter of the resolution opposing the export, said that India, which had conducted a nuclear test in 1974 using components from Canada which had led to the current legislation, had "the worst history of any of our trading partners".

He warned that to back down at this stage would damage the credibility of the whole US non-proliferation policy, and after the result of the vote was known he commented that it would now be "practically impossible" for the United States to try to convince other countries that they should accept nuclear safeguards.

This view, however, was directly challenged by Senator Frank Church, chairman of the Senate Foreign Relations Committee. Reflecting the Administration's own position, Mr Church said that not permitting the export of the nuclear fuel would abrogate an agreement reached in 1963 to supply the fuel. This would permit India to disregard other safeguards built into the agreement and could postpone indefinitely further negotiations with India on nuclear issues.

After the vote, a spokesman for the Carter Administration said that the result would help discussions with India about bringing all India's nuclear facilities under international safeguards, but there was some scepticism in New Delhi. A spokesman for the Prime Minister, Mrs Indira Gandhi, said that her government had not changed its position on comprehensive safeguards, which it still regarded as discriminatory since the major nuclear states — including the United States — were not required to open all civilian and nuclear facilities to international inspection.

This debate is not likely to go away. The Indian Foreign Ministry announced two weeks ago that it had applied earlier in September for a new export licence for 19.8 tons of enriched uranium for the Tarapur plant — a move which could stir up the whole stormy debate again.

David Dickson

Information technology

Cheering on UK

The past week has been remarkably active for British thinkers on government policy on information technology, the technology which interfaces computers and telephones, and for some of the designers of the technology itself. The Advisory Council on Applied Research and Development (ACARD), an advisory body to government and counted amongst the thinkers, published a report on the subject; the government announced some changes in the organization of responsibility for it; and British Telecom's research centre at Martlesham Heath, a designer of information technology, opened up its laboratories for more or less public scrutiny.

Central to this activity seems to be an awareness that Britain needs to put more coordinated effort into adopting information technology if it is to be competitive among the industrialized nations not only in the technology itself but also in many aspects of industry and business. This feeling certainly seems to be the basis of the ACARD report, which is more an exercise in publicity for information technology than a detailed analysis of its potential. Written by six industrialists, a trades unionist and a member of ACARD, it is an exhortation to government to take note of information technology rather than a guide to how policies might be implemented.

The report's main recommendation, that one minister and government department should be responsible for coordinating information technology policy, has to some extent been pre-empted by the government's announcement. The former computer systems and electronics divisions of the Department of Industry is to become the information division. It will assume responsibility for government policy formulation on information technology, under one deputy secretary who also has responsibility for the Post Office and satellite communications. In future, responsibility for coordinating government policy will lie with an official committee of the Cabinet Office especially set up for that purpose.

In making an international comparison of the development and uptake of information technology, the ACARD report is not very encouraging for Britain. It finds that the French, West German, Japanese and American governments are spending more than Britain on research, development and promotion. It is particularly nonplussed by the French government which, it says, has succeeded in creating considerable public awareness of information technology through one or two innovative public demonstration projects and at the same time has successfully promoted in other countries the idea that it leads the world in the field on the basis of

projects that are not yet operational. This impression has been achieved, says the report, through the skillful use of publicity.

The report does not suggest that Britain should follow suit by committing more public funds to research. However, it does suggest that a leaf could be taken out of France's book by setting up demonstration projects to increase public awareness. Suggestions for doing this include putting telex and facsimile transmission machines in Post Offices, putting Prestel receiving machines in schools and public libraries and providing telephone subscribers with black and white Prestel-like video terminals for directory information.

The government could also help develop information technology industries by buying equipment for its own departments — a move which would need to be carefully implemented to avoid creating artificial markets. The report does not mention this possible pitfall, but it does recommend that the government should help stimulate closer cooperation between users and supplies.

Despite the lack of public funds spent on information technology, Britain is by no means behind on all counts, a fact which was evident at last week's open days at British Telecom's research centre. In such an international business, however, there is strong competition between countries to have their own systems adopted as standard and Britain, says the ACARD report, has been particularly bad at promoting its own interests. It says that Prestel is suffering because extensive negotiations over viewdata standards have failed to get it accepted as an international standard despite its technological lead.

Moves should be taken, it says, to encourage pupils at school to enter this field and to encourage industry to provide its own training schemes. **Judy Redfearn**

Bulgarian science**Reaching out****Varna**

An unusual approach to international cooperation lies behind the Third PCITRRA International Colloquium (Physical and Chemical Information Transfer in the Regulation of Reproduction and Ageing), now taking place in Varna, Bulgaria. By presenting the work of young Bulgarian researchers side by side with papers by leading international experts in the field, the organizer, Dr Julia Vassileva-Popova of the Laboratory for Rapid Spectroscopy and Biological Physics of the Bulgarian Academy of Sciences, hopes to stimulate fruitful international working visits.

The theme of PCITRRA, as an international and interdisciplinary colloquium, was selected by Dr Vassileva-Popova in the early 1970s on the grounds that to compete in genetics would be to

attempt to break into a very crowded field. She felt there would be more elbow room in the area of non-genetic information transfer, the first stages of hormone action, and bioreceptor and neurotransmitter processes. Since her laboratory is more than self-financing, exporting sophisticated rapid-spectroscopy equipment primarily to the USSR, the funding of basic research is a smaller headache to her than to some other Bulgarian laboratory directors. Accordingly, Dr Vassileva-Popova has been able to gather together a large and keen team. Since East European etiquette normally calls for the laboratory head or research supervisor to be cited as a co-author, this meant that out of the 117 scheduled communications, Dr Vassileva-Popova was named as co-author of 23.

The third PCITRRA colloquium (they have been held at three-year intervals since 1974) was formally part of the celebrations of next year's 1,300th anniversary of Bulgarian statehood, and was in part supported by the State Committee for Culture — the first time apparently that this body has sponsored an international scientific event. Yet Dr Vassileva-Popova is adamant that she is not trying to found a "Bulgarian school" of biophysics/biochemistry. In her opinion, it is scientists and not their countries which matter. Indeed, had it not been for a small organizational error, she said, the participants' badges would not even have shown their countries' names. She even suggested to the international organizing committee that the fourth PSITRRA colloquium might be held outside Bulgaria — a proposal which they emphatically rejected.

Certainly, Dr Vassileva-Popova has been able to attract to Varna an impressive international gathering — including Henry Arnstein and J. R. Tata from the United Kingdom, Oscar Hechter and Elwood Jensen from the United States, Friedrich Jung from the DDR, Rostislav Sovicka from Czechoslovakia, Pushpa Bhargava from India and Andrei Tchernitchin from Chile. And this in spite of the relatively long duration of the colloquium (seven days, with working sessions of up to ten hours a day) — Dr Vassileva-Popova eschews poster sessions, and insists that all papers from her young collaborators which reach the required standard are read in full. In addition, all those foreign visitors who could spare the time were invited to stay on for a new feature of the PCITRRA colloquia, a summer school for young scientists at Burgas, further down the coast.

One notable figure was absent — Sir John Kendrew, who was detained at an EMBO meeting in Heidelberg. His encouragement of Bulgarian biophysics and biochemistry was, however, recognized earlier this year, when he was given the highest award of the Bulgarian People's Republic — the Order of the Horseman of Madara.

Vera Rich

CORRESPONDENCE

DNA X-ray data

SIR — We have recently suggested¹ that the available X-ray diffraction data from B DNA are consistent with a model for the structure of DNA which we have called the "side-by-side" model and in which DNA strands are linked together in pairs of the Watson-Crick type but are not wound into a continuous right-handed helix. Our model has however been alluded to^{2,3} in terms that suggest that alternatives to the double-helix model have been finally disposed of. We wish now to emphasize that existing X-ray data on B DNA are not in themselves sufficient for an unambiguous decision for or against either model.

Naturally occurring specimens of B DNA do not form single crystals of good quality, and the resolution of available X-ray data is very limited. For these reasons, and because there is rotational disorder in all specimens of B DNA so far reported, the only standard crystallographic quantity that can be immediately calculated from the X-ray data is some cylindrical average of the Patterson function, which must in practice be compared with the corresponding function calculated from molecular models.

We have shown¹ that in the analysis of B DNA data, there are advantages in using what we call the axial Patterson function (essentially the cylindrically averaged Patterson function with the radial coordinate zero). Briefly, the axial Patterson function is less sensitive than the Patterson function itself to imperfections of diffraction data. Of necessity, the axial Patterson function, which averages out much of the fine detail inherent in the observed diffraction patterns, can only be used for preliminary tests of the agreement between model structures and the X-ray data.

The nub of our argument is that the axial Patterson function computed from the data agrees (within the apparent experimental error) with that calculated from the side-by-side model but not with that computed from the double-helix model refined by Arnott and Hukins⁴. We do not of course claim that the double-helix model can therefore be dismissed — the quality of the data is so poor. But in the circumstances one wonders about the value of sophisticated refinements of the double-helix model (for example, ref. 4).

We are also surprised at the recent claim by Greenall *et al.*⁵ that the double-helix model is in much better agreement than the side-by-side model with the diffraction patterns measured from paracrystalline B DNA specimens. We find roughly equal disagreement for both models. (We do not wish to make too much of what we feel is an anomaly of the double helix on the tenth layer line, because it could well be an artefact of excessive refinement based on inadequate data.)

For such reasons, it must be concluded that available X-ray data are at best an equivocal means of deciding between alternative DNA models. So much is acknowledged by Crick *et al.*⁶. For X-ray analysis to provide unambiguous distinction between different models of DNA, it will be necessary for much better crystals to be prepared from well characterized polynucleotides. In the meantime, while no doubt the merits of alternative DNA models will be assessed by considerations other than those deriving from

X-ray data, it is prudent that the present insufficiency of the latter should be widely and openly acknowledged.

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R.H.T. BATES
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Andrei Sakharov

SIR — The Olympic games are over; the hopes that Academician Andrei Sakharov might be allowed to leave his internal exile in Gorky and return to Moscow are over now too. Professor Sakharov has been exiled for over nine months and has no contact with his scientific colleagues, is not allowed to take part in seminars or lectures, has no access to scientific information so crucial for his research. (Books and journals have to be brought to him by his relatives from Moscow, which usually takes weeks or months. Since January, his colleagues from Lebedev Institute have been allowed to visit him only three times!)

In spite of this, he has managed to write three scientific papers in Gorky, and English translations have been published at SLAC, Stanford University (Estimate of the Quark-Gluon Coupling Constant; Cosmological Models of the Universe with Rotation of Time's Arrow; Mass Formula for Mesons and Baryons).

I am convinced that it is time now for the world scientific community to escalate the efforts to help our distinguished Soviet colleague. From Andrei Sakharov's last communication it is obvious that he is mainly missing the information about what is going on in physics.

It should be very easy for the physicists to break down this information barrier. Let the theoretical institutes, laboratories and groups from all over the world begin sending to Professor Sakharov their preprints, lecture notes, reports. Send them by registered mail with the red "Avis de Reception" card to:

Professor Andrei Sakharov
Prospekt Gagarina 214, kv.3
Scherbinka 2, Gorky, USSR

Don't hesitate to ask your local post office to investigate if the red card is not returned to you with Andrei Sakharov's signature within about a month. Your local post office is obliged, according to international postal convention, to make the investigation and, if unable to provide proof that the mail was delivered, to pay you compensation.

There is no doubt that such world-wide action will not only provide Professor Sakharov with necessary scientific information but will also represent considerable moral support for him. It will be important for Soviet authorities too: they will see that Andrei Sakharov's case is not at all forgotten by the world scientific community.

F. JANOUGH

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Preventing plagiarism

SIR — The article "An outbreak of piracy in the literature" (*Nature* 12 June, p.429) shocked me deeply. In December 1978 Dr Alsabti sent manuscripts to us also, with the headed paper of the M.D. Anderson Tumor Institute. Again, he gave a private address in Houston for correspondence and made acknowledgement to the Jordan Royal Family. Fortunately for us, he grossly overdid it, sending five manuscripts within a month. A call to M.D. Anderson helped us in taking the decision to reject the five manuscripts.

An old custom in the Italian universities called for the approving signature of the professor on the front page of a manuscript. Although I do not agree with a system which may impair scientific freedom, the present incident should teach us how important it is to ascertain directly from the host institution the credentials of an intended author, particularly when complex international relationships are apparent.

UMBERTO VERONESI

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Pesticides as poisons

SIR — Peter Clarke (*Nature* 18 September p.184) defines a poison, but fails to note that "the dose alone determines the poison" (Paracelsus, 1564), a point that was emphasized correctly in *Nature* 28 August p.832. He says high blood levels of DDT in Guatemala were "the corollary" of an "aggressive marketing campaign" by a transnational corporation, and that cigarettes and pesticides are "harmful products". Cigarettes are harmful to human beings, pesticides to pests. The use of DDT in Guatemala was started by the World Health Organization and the United Nations International Children's Emergency Fund to control endemic malaria in 1,500 Guatemalan communities. A reduction in malaria was achieved.

THOMAS H. JUKES

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Berkeley, California*

SIR — Of necessity, I have had to become something of an "applied toxicologist".

The only still-unfalsified definition of a poison is that of Paracelsus "*Dosis facit venenum*"; the definition offered by your correspondent Peter Clarke (*Nature* 18 September, p.184) contains less of the essential nature of a poison, and therefore fails. *Nature*, consequently, has avoided polemic (unlike your correspondent), and has indeed abandoned prejudice. Dr Clarke's letter is a brilliant piece of special pleading on behalf of a political stance — and I congratulate *Nature*, Sir, on your stand for true objectivity.

I am about to retire from full-time employment as a scientist, but I hope to maintain an attitude of "informed scepticism".

R.A. DAVIS

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NEWS AND VIEWS

Myths, models and human evolution

What on earth can safely be said in public about human evolution? The charitable among those who heard Dr J. R. Durant's Darwin lecture to the British Association this year will have been worrying at this question for the past three weeks. The title of Dr Durant's address — The Myth of Human Evolution — tells the tale sufficiently: the stories that people tell, he argued, and which have been used for various purposes by people as different as Freud and Galton, Lorenz and E.O. Wilson, are but myths; "theories of human evolution have come to embody contemporary hopes and fears about the society in which we live". Specifically, the accusation goes, since the time of Raymond Dart until quite recently, the story told of human evolution has involved a model of human nature which is consistent with the doctrine of Adam and Eve, the Garden and the Fall, the doctrine of Original Sin, the notion that each contemporary human being is a battleground between civility (in the strict sense) and the primitive and powerful Beast within. The notion that primitive men were not merely successful hunters of other species but killers of each other, Durant says, fitted well with contemporary views of society at war within itself. But now, says Dr Durant, the fashion is changing. In making the public legend of human evolution, people have taken to describing primitive humans or hominids as creatures whose survival depended on cooperation between individuals and among groups, whose behaviour was shaped by an implicit recognition of the benefits of the division of labour in a hunter-gatherer society which Adam Smith first clearly described, and whose distinctive attribute — that of language — predisposes towards communality. What view, it might innocently be asked, is right? Neither, said Dr Durant. Each is a myth. Tell us instead, he asked of palaeoanthropologists, palaeoarchaeologists and the rest, the truth. Or at least acknowledge that your data are insufficient to say very much at all.

Stirring stuff like this is certain to evoke some kind of a response. Dr Durant will not have been surprised that, to begin with, the British daily newspapers were hesitant in knowing where he stood. Was he an anti-Darwinist, a Lamarckian or perhaps even a Creationist? When the fine print showed that he is none of these things, the tide of excitement ebbed. Mr Ronald Reagan, running for President of the United States, had in any case captured more attention with his aside in Dallas, Texas on the teaching of evolution in American schools (and may yet capture the Creationist vote in the United States with a sufficiently well-calculated goof.)

Durant's complaint, in any case, was a complaint against a style of argument. His targets were writers such as Lorenz (in the old school) and E.O. Wilson (in the newer fashion). His audience, given its provenance, may not fully have appreciated that Durant had selected pushovers for his attack. "Criticize Lorenz's concept of innate aggression" must now be a standard essay topic in undergraduate biology courses in most universities. Before long it will be joined by topics such as "What is the evidence (if any) for the belief that genetically determined altruism has played a part in human evolution?". He was entirely within his rights to complain that, in much of what passes for a general account of the evolution of human beings and of human nature, people who should know better are too fond of making too much of far too little evidence. Their common error is to take a living primate species as a model for earlier — and even contemporary — human (or hominid) society. Human nature being what it is, it is not surprising that these reconstructions reflect their prejudices or their hopes of wish fulfillment.

So far, so good. Dr Durant must have been well satisfied with

his attack on such a variety of false prophets. Yet there is more than this to say and to ask. Why, for example, does the question of human evolution evoke such powerful interest, and such passion? What is to be said with confidence in such a field, where the data are likely always to be insufficient? And how should an account of human evolution be constructed — and made immune to the attentions of the myth-makers?

Nobody should be surprised that these issues excite deep and general interest. Long before Darwin made it possible to talk about the issues in concrete terms, people at large were understandably preoccupied with the subject matter of *Genesis*. All writers on the human condition have found it necessary to tackle the same questions — although it remains a puzzle that the Greeks, typified by Plato in the *Laws*, contemplated a past more or less indistinguishable from their present. For the past century, however, the public interest in human evolution has mostly been guided by Darwin's terms of reference. It would have been charitable of Dr Durant to have acknowledged that that is a step forward, and away from myth. But there is, as he complained, some way to go.

The lack of evidence is the chief stumbling block. The palaeoanthropologists may not really agree, but they are fortunate among those who work on human evolution. They have bones and other objects to measure, but past behaviour has left no fossils, only occasional artefacts from which behaviour must be inferred. In the circumstances, it is remarkable how much has been accomplished with the fossils uncovered in the past half-century. The time-span of human and earlier hominid evolution has been pinned down more accurately than could have been hoped a decade ago: 3.25 million years, give or take the usual ten per cent, will span the earliest australopithecine so far known (*A. afarensis*) and the present. Anatomical evidence appears to make possible the distinction between potential ancestors of hominids and apes among the contemporaries of late-Tertiary *Ramapithecus*. The palaeoanthropologists have been helped along by pieces of unexpected good luck — such as the way in which genetic differences between extant primate species will increasingly be used as evolutionary clocks. Even so, the reconstruction of this relatively tangible part of human evolution is full of uncertainty. Was *Homo habilis* an ancestor, or a dead end? Which of the specific names which the palaeoanthropologists have put into circulation will survive discoveries not yet made? Calculated scepticism — which jibes uneasily both with the temperament of the practitioners and the expectations of the public — is clearly essential.

So how, with such caveats, can progress be made towards a robust understanding of how early people, and hominids, behave? Most of Dr Durant's complaints were levelled at those who have embellished fossils with patterns of behaviour for which there is no evidence. He may have unfairly overlooked the opportunities that do exist for tackling these questions empirically — for example, by constructing models of the coordinated evolution of anatomy and behaviour, using data gathered not merely from the fossil beds but from analyses of the structure of, say, intelligence, observations by psychologists of the acquisition of specified skills in infants and other primate species and even the evolutionary implications of, say, protein biochemistry.

The trouble, of course, is that models are easily misunderstood. The safeguard, in this field as in, for example, cosmology, is that their function should be widely understood. A model is but a hypothesis to be tested. To regard it otherwise is to make a myth of it.

Infrared astronomy comes of age

from R. D. Wolstencroft

A recent conference* provided the first opportunity to discuss the contributions to astronomy being made by the new infrared telescopes on Mauna Kea, Hawaii. Infrared observations, both of cool clouds of interstellar gas and of the infrared sources within them, give vital information on the conditions under which star formation occurs. A wealth of new information is being obtained by a range of sophisticated instruments and the first studies are being carried out in regions of star formation outside our Galaxy.

Interstellar clouds exhibit a wide range of size, mass, density and temperature. They are observed over a wide range of wavelengths, from the optical to the radio, and with a variety of techniques which emphasize regions of differing density and temperature in a given cloud. The synthesis of such observations to give an overall picture of a cloud are still at a rather rudimentary stage. Because of this and also because of the continuous nature of the cloud — inter-cloud medium no completely logical classification for clouds has yet been derived. Thus the terms in use such as 'molecular cloud', 'dark cloud' or 'globule' do not necessarily define objects with similar physical properties. R. J. Evans (University of Texas) summarized the properties of molecular clouds and discussed the problems of classification. A typical giant molecular cloud is 100 pc across with a mass, up to half of which may be molecular hydrogen, of about 10^5 solar masses. Stars tend to form in the densest and hottest parts of the cloud, usually in clusters. Massive stars form preferentially in the larger clouds whereas the low mass stars form in clouds of all sizes. The infrared sources found in the molecular clouds may be either embedded stars or protostars; they are very luminous, typically in the range 10^3 to 10^5 times the solar luminosity.

Clouds at the low end of the mass and length scale are the Bok globules which were discussed by A. R. Hyland (Mount Stromlo Observatory). A promising new approach was described for studying the short wavelength infrared extinction by looking at background sources seen through a globule. The technique has been applied to a relatively symmetric globule in the Coalsack dark cloud. From the canonical relation between infrared colour excess, visual extinction and molecular hydrogen column density, a mass of 11 solar masses and a radius of 0.25 pc can be derived.

R.D. Wolstencroft is at the Royal Observatory, Edinburgh.

Thermal emission from the dust at far infrared wavelengths can yield the typical temperature (about 15° K) and mass (about 0.1 solar mass) of the dust in a globule. It is probable that most globules are not undergoing gravitational collapse.

The Orion Molecular Cloud (OMC) is a well studied region which received much attention at the symposium. N. Z. Scoville (University of Massachusetts) described a model of an expanding envelope of gas centred on the Becklin-Neugebauer (BN) and Kleinmann-Low complex of infrared sources: the expansion velocity, based on CO mm line measurements, is 50 km sec⁻¹ at 0.1 pc from the centre. A quite different view is held by R. Genzel (Center for Astrophysics) who proposed a model based partly on a VLBI proper motion study of maser sources: the source IRC 2, rather than the BN object is the centre of a low velocity (18 km sec⁻¹) outflow with a higher velocity outflow in the surrounding region. IRC 2 is an unusual source: it has a very strong 10 μ m absorption feature, possibly a greater luminosity than the BN source and it is the only SiO maser in a star-forming region. Other evidence of energetic motions in OMC comes from observations of molecular hydrogen emission in the 2.1 μ m vibrational quadrupole line and other rotational lines: this emission may be due to shocked gas at a temperature of about 2,000 K.

Results from a variety of sophisticated instruments were presented, notably those in the field of far infrared spectroscopy and speckle interferometry. As an example, infrared speckle interferometry by R. R. Howell and colleagues (Steward Observatory) has shown that the source W3 IRS 5, previously thought to be one source, is in fact a double source with a one arc second separation. If many sources prove to be double or multiple and if the components were to have different temperatures this could significantly affect our interpretation of source temperatures. F. Sibille and P. Lena (Meudon Observatory) have measured upper limits to the angular size of several sources, including 0.08 arc s for BN at 2.2 μ . There is a strong possibility that proto-planetary disks will eventually be resolved by this technique.

Evidence for the presence of magnetic fields in molecular clouds comes from recent studies of the polarization of infrared sources in the clouds, a topic reviewed by H. M. Dyck and C. J. Lansdale (University of Hawaii). The generally large linear polarization, the approximately constant ratio of circular to linear polarization and wavelength dependence are consistent with a model of aligned grains in the vicinity of the source. An alternative explanation for the observed polarization in terms of

scattering by grains in a bipolar nebula or similar geometrical configuration appears to be ruled out. A twist in the grain alignment along the line of sight near the source is implied by the circular polarization: twists of between 20 degrees and 80 degrees can be deduced for the nine sources so far studied. The grains are most probably aligned by an enhanced Galactic magnetic field permeating the cloud. Questions concerning the strength of the field, the origin of the twists in the grain alignment and the location in the cloud of the polarizing region, are of great interest and should be answered by further careful polarimetric observations in the infrared.

The latest observational results concerning the galactic centre were reviewed by I. Gatley (UKIRT, Hawaii) and E. Becklin (University of Hawaii). Outside the central few parsecs an expanding ring of molecular clouds has been identified from CO mm line measurements. The far infrared luminosity (30 to 100 μ m) in the region can be explained by thermal emission from dust heated by a large density of late type stars. Within the central few parsecs are a number of 10 μ m sources which appear to be objects a fraction of a parsec across with about one solar mass and 10^5 solar luminosities. A preliminary analysis of the observations suggests that they may be losing mass at a high rate: the nature of these objects is quite uncertain, but it is tempting to think of them as analogues of the BN object. Another important finding, based on 30 to 100 μ m observations, is the low abundance of dust within the central parsec. A black hole may be needed to explain the variety of observational results now available.

Star formation in the nuclear region of external galaxies was reviewed by G. H. Rieke (University of Arizona). About 40 percent of galactic nuclei so far studied show a large infrared excess at 10 μ m: whenever this excess is seen a very large infrared luminosity (10^9 to 10^{10} solar luminosities) is also seen within a few hundred parsecs of the centre. The 10 μ m silicate feature is also present in these same galaxies. Rieke argues that thermal radiation by dust is the immediate source of the observed radiation with the ultimate origin of the high luminosity being rapid star formation in the nuclear region. The nuclear infrared source is resolved in all cases. For example C. T. Telesco (University of Hawaii) reported that the 10 μ m emitting region of M51 has a diameter of about 3 kiloparsecs. Rieke posed three important questions which should be addressed in the near future: (1) What is the initial mass function (IMF) for star formation in nuclear regions? From a study of M82 it appears that there may be a bias against the formation of stars with masses below 2 or 3 solar masses relative to

*The International Astronomical Union Symposium on Infrared Astronomy was held in Kailua-Kona, Hawaii, June 23-27, 1980 and was sponsored by four organizations operating telescopes on Mauna Kea: NASA (3.0m Infrared Telescope Facility — IRTE), SRC (3.8m UK Infrared Telescope — UKIRT), the Canada-France-Hawaii Telescope Corporation (3.6m CFHT) and the University of Hawaii (2.2m UHTE).

Hurricane Allen destroys Jamaican coral reefs

from J. D. Woodley

HURRICANE Allen, which traversed the Caribbean in early August, was the second strongest ever recorded with wind speeds approaching 300 kph (185 mph). Fortunately, its track was mostly over the sea but, while the islands were spared the impact of extreme winds, they were exposed to enormous storm-generated waves. In the small hours of Wednesday 6th August, Allen passed within 50 km of the north-east coast of Jamaica. Severe wind damage occurred to the forests of the Blue Mountains and the banana plantations of the adjacent lowlands. Huge waves beat upon the eastern and northern shores, claiming several lives. Houses built on cliffs 12m above the sea were destroyed. Beneath sea-level, the spectacular fringing coral reefs suffered considerable damage.

The Discovery Bay Marine Laboratory of the University of the West Indies is at a site in the middle of the north coast and not in the area hardest hit but, after the hurricane waves subsided, the water was turbid with fine sediment and pulverised tissues; there was a smell of dead corals, gorgonians and sponges. Death and injury to these and other organisms have now been assessed. Branching corals have been smashed, massive corals toppled or split, and plate-like ones fractured or torn off the reef slopes. Gorgonians and sponges have been ripped away or broken, echinoids destroyed and many fishes injured too. Physical damage varies with exposure and submarine profile, but in some places extends below -50m. Tons of sediment have been removed from the reef slopes and terraces. Sand-blasting and flying missiles have caused extensive abrasion, especially in the shallowest zones, where almost total destruction prevails. Before the storm, luxuriant stands of elkhorn coral (*Acropora palmata*) dominated the breaker zone from 0 to -6m (Fig. 1). This species is well able to resist the forces of ordinary breaking waves, but evidently not those generated by a severe hurricane. It has been reduced to rubble (Fig. 2) and some of this flung on to the reef flat, forming islands where none was before.

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Fig 1 A stand of *Acropora palmata* in the breaker zone before the hurricane.

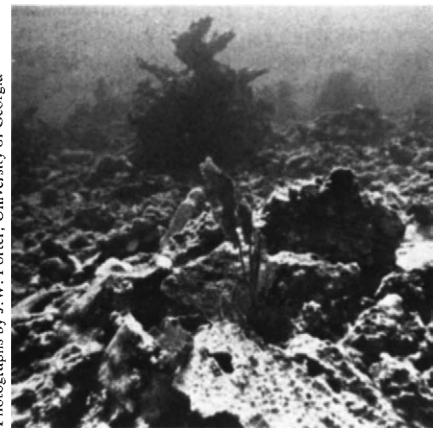


Fig 2 The breaker zone at the same locality after Hurricane Allen.



Fig 3 A diver (K. W. Rylaasdam) photographing newly exposed substratum on an over-turned head of *Monastrea annulans* at a depth of -11m. More bare substratum can be seen where some 30cm of sand was removed by the hurricane. In the background are smashed colonies of staghorn coral, *Acropora cervicornis*.

Studies are continuing on the survival of broken or disturbed corals and other sessile organisms, the healing of injuries, changes in demersal plankton, changes in the reef-fish community and the colonization of bare substrata. The last of these, beginning the long process of regenerating a new reef community, is of particular interest. Abrasion, fracture and sediment removal have exposed large areas of new surface for colonization throughout the reef profile (Fig. 3), but especially in shallow water.

Already, there are massive blooms of various green algae. Will the subsequent communities resemble the pre-existing ones, and how long will they take to develop? In most of the reef zones at Discovery Bay, the disturbance was spectacular but not totally destructive; recovery is likely to occur within a few years. Where extreme damage has occurred, that is, in shallow water and progressively deeper on reefs further east, it is possible that 'recovery' will take decades.

the IMF for the solar neighbourhood. (2) Are there any galaxies intermediate between the high and low infrared luminosity galaxies? (3) What mechanism starts and sustains the star formation activity?

The correlation between neutral hydrogen velocity width and integrated magnitude for galaxies (Fisher-Tully relation) can be used to determine the Hubble flow velocity, H_0 , and hence the extragalactic distance scale. M. Aaronson (Steward Observatory) discussed his latest results using infrared magnitudes which

give a tighter correlation than the optical magnitudes used in earlier studies. With the exception of galaxies in the Virgo cluster the value of H_0 is $95 \pm 4 \text{ km sec}^{-1} \text{ Mpc}^{-1}$. Judging from the reaction to Aaronson's talk at the meeting this new value of H_0 is being treated very seriously. Since the canonical value of $55 \text{ km sec}^{-1} \text{ Mpc}^{-1}$ by Sandage and Tamman is very much less, it is clear that the origin of the discrepancy between these values must be urgently sought. For Virgo $H_0 = 65 \text{ km sec}^{-1} \text{ Mpc}^{-1}$ which can be interpreted as an infall of the local group of galaxies towards

Virgo at a radial velocity of 480 km sec^{-1} .

The vigorous growth of the subject makes it likely that this first symposium devoted to all major fields of infrared astronomy may well be the last with such a wide compass. The direction this expanding field of research will go in is not easy to predict. However it is clear that infrared observations of redshifted galaxies will play a vital role in the solution of many of the most important cosmological problems, a point stressed by M. Longair (University of Cambridge) at the end of the symposium. □

Industrial biopolymers

from Paul Calvert

THE term biopolymers really includes all macromolecules of biological origin but, for industrial application, biopolymers can be divided into two categories: those to be used in aqueous solution and those to be used as solids.

The first category contains a wide range of polysaccharide thickeners, emulsifiers and gelling agents used in the food industry and in a wide range of printing and coating applications. These materials can be extracted from trees, seeds and seaweeds or can be manufactured by modifying starch or cellulose. A variety of microorganisms also produce water soluble extracellular polysaccharides and it was a microbial product — Xanthan gum — that was the centre of attention at a recent meeting in Birmingham*. Xanthan gum is produced commercially by Kelco from *Xanthomonas campestris* and consists of a glucose main chain with three-unit side chains (mannose-glucuronic acid-mannose) on every other glucose residue. It was picked for its unusual gelling properties from a survey of microbial polysaccharides carried out by Allene Jeanes at the US Department of Agriculture in the 1950s. It is pseudoplastic, that is it forms a gel or high viscosity solution which thins when the solution is stirred. The viscosity is stable to changes in temperature, pH and salt concentration but can be modified by adding other vegetable gums. This gives it a wide range of uses, for example in 'instant' desserts and in salad dressings that flow out of the bottle but not off the salad.

One very important possible application for water soluble polymers is in the secondary recovery of oil. A normal oil well only produces about 40 per cent of its oil content in the combined processes of primary recovery, where the oil comes out under its own pressure, and secondary recovery where it is forced out by water or gas pumped down a series of holes surrounding the oil reservoir. The extraction is limited by the failure of the water to displace oil from porous rock and by the tendency of the water to follow cracks in the rock directly to the central well rather than sweeping the oil before it. There are a variety of ways to enhance oil recovery (for a review see Arnold, C.W. in *Microbial Polysaccharides and Polysaccharases* ed. Berkeley, Gooday, & Ellwood, SGM/Academic Press, 1979) but a possible new approach is through a combination of surfactants, to allow the water to displace the oil from rock, with water-soluble polymers to increase the water viscosity and prevent it by-passing the oil. The requirements for such a polymer are that it gives a high viscosity increase at low concentrations but is shear-thinning for ease of pumping. It should

also be stable at the bottom of an oil well for several months, which, in the case of deep North Sea wells where conditions are most severe, means it must be stable in saline at temperatures of about 120°C. Polyacrylamides have been used successfully in the US although the cost was high compared to the value of the oil produced. For general use, polyacrylamides are too unstable and too easily absorbed and every known water soluble polymer has been considered as an alternative. Xanthan gum or a similar microbial polysaccharide seem to be suitable but current fermentation methods of production make them expensive. Also, at a rate of one pound of polymer used per barrel of oil produced there may not be sufficient production capacity for the polysaccharides to satisfy the potential demand even if all fermentation plants were employed for this one product.

In the light of the increasing price of oil and the current interest in fermentation methods of producing industrial chemicals it seems natural to seek an industrial plastic made by microorganisms. For a biopolymer to be used as a plastic it should be a

*A symposium on "Recent advances and Industrial Applications in Biopolymers" organised by the Society of Chemical Engineers was held in Birmingham in March

linear uncross-linked molecule insoluble in hot water but soluble in organic solvents or, better, capable of melting and processing without decomposition. Given that natural structural polymers like cellulose and collagen tend to be made from polar monomers and derive their insolubility from strong intermolecular hydrogen bonding it is not surprising that suitable examples are rare. One example is poly (β -hydroxybutyrate) which is an optically active thermoplastic polyester with a melting point around 175°C produced as an intracellular storage compound by various bacteria including *Azotobacter vinelandii* and *Pseudomonas solanasearum*.

If a suitable naturally produced thermoplastic were available it may still not be practicable to manufacture it. Any fermentation process leads to products which must be separated and purified and this may be considerably more expensive than a simple catalytic polymerisation, (for example, ethylene to polyethylene) although it may be competitive with the more expensive condensation processes leading to nylons or polyethylene terephthalate.

Thus while the traditional biopolymers, cellulose and rubber, are still the most important and water soluble biopolymers are in production, a solid polymer, the plastic microbe, has yet to come. □

Essential fatty acids and prostaglandins

from Michael Crawford

IN 1930 at the University of Minnesota in Minneapolis, George and Mildred Burr made the remarkable discovery that dietary linoleic acid was essential for growth, development and normal function in rats. At this time, pharmacological activity of a group of uncharacterised substances from semen and male accessory glands was also being studied by U.S. von Euler at the Karolinska Institute in Stockholm. He characterized the material as a biologically active hydroxylated unsaturated fatty acid and named it 'prostaglandin'.

World War II interrupted research on the 'essential fatty acids' (EFA) and 'prostaglandins' (PG), and it was not until the 1950s, with the introduction of radioisotopes, gas-liquid chromatography and mass spectrometry that further progress was made. During the 1950s and 1960s EFAs were shown to be required by several species, including man, and linoleic acid was found to be metabolized to longer chain polyunsaturated acids. In 1964 these were established as the precursors for the PGs by Van Dorp and colleagues of Unilever in Holland and by Bergstrom and colleagues at the Karolinska Institute in Sweden.

In spite of this evidence establishing a

link between EFA and the PGs, work in both fields remained separate. Pharmaceutical companies became excited by the potential of PGs, their analogues and inhibitors in the fields of reproductive physiology, inflammation and thrombosis. Meanwhile in the EFA camp, advances were made in establishing the role of EFA in cell membrane function, brain growth and the prevention of atherosclerosis and thrombosis. At the time, however, benefits of high linoleic acid diets to the cardiovascular system were largely attributed to reduction in blood cholesterol. The important role of EFAs and PGs may well be in the integrity of vascular membranes, the stability of platelets and in modulation of vascular tone.

A recent Congress* was held to bring the two fields together. Both Burr and von Euler attended the Congress as guests of honour. The sessions were designed to highlight the precursor-product relationship between EFAs, PGs and other biologically important EFA metabolites.

A number of different speakers independently supported the idea that nutritional management of dietary precursors should affect biosynthesis of PG and other oxygenated products. The practical applications of this include the reduction

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*The Golden Jubilee Congress on Essential Fatty Acids and Prostaglandins was held at the University of Minnesota, May 4-7, 1980.

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of hypertension and of atherosclerosis and the prevention of retinopathy in human diabetics by a diet rich in linoleic acid over a six-year period. In cardiovascular disease also, EFA aspects of nutrition emerged as a positive contribution to health in contrast to the negative effects of saturated fats and cholesterol.

It was suggested that conventional descriptions of EFA deficiency as a scaly skin and an elevated triene/tetraene ratio may be inadequate because different disease states were described in which conversion of linoleic acid to the PG precursors were impaired. Psoriasis is one example of EFA deficiency in which supplies of the linoleic and arachidonic acids are adequate but in which there is a block in the enzyme cyclooxygenase necessary for PG formation. In addition, the conversion of dihomogammalinolenic and arachidonic acids to the spectrum of leukotrienes, thromboxanes and prostacyclins may need to be similarly considered. In this context, present recommendations for linoleic acid intake of 1-2% of dietary energy may have to be revised upwards, especially when one considers our present high consumption of saturated fatty acids and their competition with EFAs for the enzyme systems. It is possible that the health of the vascular endothelium and the prevention of atherosclerosis should be a criterion by which one measured EFA requirements.

New leukotrienes were described bringing to three the families of leukotrienes. Within each family two compounds have pro-inflammatory and bronchoconstrictor roles making them possible mediators of inflammation and asthma. Probably these account for the biological activity of the slow reacting substance in anaphylaxis (SRS-A). There were also reports on chemical synthesis of novel leukotrienes and of the biosynthesis of leukotriene-like substances from 18-carbon fatty acids possessing Δ 5-unsaturation.

In the prevention of thrombosis and control of the immune system, there was renewed speculation that PGE_1 and/or other derivatives of the EFA precursor (8, 11, 14-20:3) may play previously hidden roles. Alteration of PG metabolism induced by the therapeutic use of lidocaine in endotoxin shock in baboons indicated that prostacyclin might be involved in protection against shock. This finding, for the first time, provided a rational link between adequate nutrition and susceptibility to stress and shock.

Columbinic acid proved to be of great importance. Derived from the seeds of the Columbine, its structure is similar to linoleic acid with a *trans* double bond at the five position. This acid was found to express many EFA functions associated with linoleic acid, but it cannot be converted to prostaglandins. Columbinic acid thus provides an experimental tool for separating PG-mediated functions from

the structural lipid functions of essential fatty acids. For instance, columbinic acid could restore skin lesions of EFA-deficient rats, but could not normalise the response to inflammation.

Other exciting topics included a report suggesting that arthritis may be alleviated by dietary means involving 6, 9, 12-18:3. This result was found in an animal model of immune-mediated arthritis, the leukocytes from such animals were not able to take part in chemotaxis. With this and the other reported examples from the immune response and cardiovascular areas, the distinct impression was given that we may be on the verge of new and exciting developments in dietary control or prevention of major chronic disease states.

The Congress banquet ended with two

historic speeches from two remarkable men — Burr and von Euler — remarkable not only for their work but for their great insight and natural humility. As scientists we were reminded to “expect the unexpected” and to realise that original discoveries often only become important after development by others whose contributions are forgotten.

The unique formula for this Congress worked in a very special way. It was the first meeting of two branches of science which have been distant acquaintances for many years; the knowledge each offered the other fused them into one. It is to be hoped that the new links and friendships it created will help to expand the new and permanent dimension that was added to biology in Minneapolis.

Neural communication and control: facts and theories

from D. M. MacKay

Everyone would agree that the interaction between theory and experiment should ideally take place in one and the same head. This is doubly true in a field such as brain research, where we are often in doubt as to the categories in which our problems should be expressed, let alone resolved. Present-day education being what it is, however, few of those most productive in experimental neuroscience are ideally equipped to do their own theorising; and all too many theorists show a lack of that sense of proportion that comes from long exposure to experimental fact.

Perhaps because of its close kinship with problems of engineering, the study of neural communication and control has enjoyed more than its share of theoretical attention over the past three decades. Can we learn anything from the history of those exceptional theories that have succeeded in catalysing experiments on new lines with useful results? Conversely, which current experimental findings (if any) seem likely to set theoretical speculation off in fresh directions? These were the kinds of question faced at a recent Symposium*.

T. H. Bullock (University of California, San Diego) set us off with an impressive survey of the diversity of physical changes that might serve to represent information or to control function in a single neuron, and therefore should not be overlooked by theorists of neural communication. Field potentials can be causative (for example, at the Mauthner cell axon cap). The relative timing of impulses may be as important as their mean frequency, especially if central ‘clocking’ occurs. Even physical movement of fibres cannot be discounted. Much of the ensuing discussion sought to narrow the range over which these

alarming complications may be significant; but there was general agreement that at least in plexiform tissue the representation of neuronal interaction by means of ‘circuits’ was too simple to be safe.

Simultaneous recording from multiple microelectrodes, described by G. L. Gerstein (University of Pennsylvania) and others, offers a particularly promising new line of data for theorists of local network function. With tungsten microwire bundles, for example, Gerstein has found much greater similarity in the patterns of coincident firing of a group of visual cortical neurones than in the responses of a single neuron to two successive stimuli. This suggests that sensory information may be represented with lower variance in ensemble statistics than in single-unit histograms. A host of theories of neural network function in other areas may become testable as this technique develops.

A beautiful preparation for the study of neuronal interaction was described by P. Andersen (University of Oslo), who has succeeded in maintaining isolated slices of hippocampal cortex in functional condition for recording *in vitro*. Among many important results, he finds remarkable equipotency of synapses on the inner regions of pyramidal cell dendritic trees, and virtual simultaneity of excitation of a row of pyramidal cells by parallel afferents. Summation of simultaneous EPSPs from neighbouring inputs is perfectly algebraic, with no sign of conductance interference even at synapse separations below 50 μm . Andersen’s recordings identify GABA-containing interneurons as important modulators of pyramidal function.

*A Satellite Symposium of the 1980 Physiological Congress, co-sponsored by the Commission on Communication and Control Processes of the International Union of Pure and Applied Biophysics, was held between July 20 and 24 at the University of Debrecen in Hungary. The organizers were S. Damjanovich & G. Szekely (Hungary), P. I. Johannesma (Netherlands) and D. M. Mackay UK.

D. M. MacKay is Head of the Department of Communication & Neuroscience, University of Keele, UK.

An elegant experiment was described by M. Constantine-Paton (University of Princeton), on the tectal innervation from a third eye implanted in the developing frog. This shows that stereotypic segregation into alternating bands of ocular dominance can develop from an initial state of overlap between the two projections, and is superimposed upon a retinotopic representation of visual space. R. M. Gaze (National Institute of Medical Research, Mill Hill), working with compound (double-nasal, double-temporal, double-ventral) eyes in *Xenopus*, reported data supporting the 'tea-trade' model of connection-formation developed by Willshaw and von der Malsburg¹.

The cerebellum has long invited theoretical modelling by the regularity of its geometry. Another highlight of the meeting was the study by M. Ito (University of Tokyo) of adaptive changes in the flocculo-vestibulo-ocular system of the alert rabbit, which has for the first time yielded a detailed and nearly complete functional picture of a cerebellar subsystem. Mossy fibre impulses from the labyrinth are transferred across the flocculus and elicit signals from Purkinje

cells that adjust the vestibulo-ocular reflex. 'Retinal slip' error signals, sent to climbing fibres via the inferior olive, modify the efficacy of mossy fibre synapses on the Purkinje cells until the adjustment is accurate. Conjoint stimulation of mossy and climbing fibre afferents reduces the efficacy of parallel-fibre synapses. Although the synaptic changes found are opposite to those postulated in Marr's theory², being rather in the direction assumed by Albus³, the general framework of Marr's original conceptualisation of cerebellar function appears to be supported by these findings.

At the theoretical 'whole-brain' level V. Braitenberg (Max-Planck-Institute, Tübingen) challenged us to consider the implications of some neuroanatomical statistics. With pyramidal cells making up perhaps three quarters of the neuronal population, synapses at a density of 10^9 per mm^3 , and fibres perhaps 3×10^9 per mm^3 ,

he argued that more than 100 axons must cooperate in order to excite one pyramidal cell. Hence the influence of any one pyramidal cell on another might be relatively small, despite their proximity. He favoured a view of the cerebral cortex as an array of associative cell assemblies rather than of feature-classifying units, with co-operative state-transitions representing specific 'decisions'.

Finally, D. M. MacKay (University of Keele) described recent experiments, based on his earlier information-flow analysis of cognitive agency⁴, which gave evidence of goal-conflict between the two 'halves' of split-brain patients only at the executive level. The inference suggested was that the direct physical correlate of cognitive agency is not cortical activity as such, but rather the cooperative activity of a 'supervisory system' whose dominant evaluative level is embodied in diencephalic or other structures not divided by the splitting operation.

One's impression from the meeting as a whole (from which the above are only samples) is that the working relationship between experimentalists and theorists of neural function is entering on a new and remarkably fruitful phase. □

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4. MacKay, D. M. in *Brain and Conscious Experience* (ed. J. C. Eccles) 422 (1966); *Cerebral Correlates of Conscious Experience*, (eds. P. A. Buser & A. Rougeul-Buser) 53 (1978).

Chopping and changing in immunoglobulin genes

from Miranda Robertson

A concluding speaker, summarizing one of the 19 themes of the 4th International Congress of Immunology*, remarked that immunological research seems not so much to rush forward like a river, as to advance and retreat by turns like the tide. While there may be some truth in this assessment for cellular immunology, it clearly does not apply to the molecular biology of immunoglobulin genes, which has been advancing with gathering momentum ever since Tonegawa and his colleagues announced the first V region sequence in 1978¹. Yet even in this field, there were signs at the Congress, if not of a retreat, at least of a significant change in orientation. Many of the molecular biologists who have been seeking an explanation for the diversity of antibody molecules have now clearly shifted their attention from somatic recombination of DNA to somatic point mutations.

The data which have led to the recent emphasis on recombination were reviewed by E. Kabat (Columbia University), and can be recapitulated as follows (see also ref. 2). It is known that the antigen-binding variable region of the immunoglobulin light chain is constructed from two separate genes: a V gene and a J gene. During the differentiation of an immunoglobulin-secreting B cell, one of a set of between 100 and 1,000 germ-line V genes combines at random, and with slight variations in the precise site of recombina-

tion, with one of a set of 5 germ-line J genes. The site of recombination coincides with the third hypervariable region. The heavy-chain variable region is constructed by a similar process with one additional complication. The V genes of heavy chains do not join directly to the J genes, but to a third set of genes, the D genes, which join in turn to the J genes. The D region of the heavy chain, which may vary considerably in length, corresponds to the third hypervariable region.

Given that each of the two immunoglobulin chains is constructed by such random recombination of substantial sets of germ-line genes, and that the light and heavy chains assort independently, it has been argued, here² and elsewhere, that there are enough germ-line immunoglobulin genes to account for the entire antibody repertoire without any need to postulate further diversification by mutation.

But whether or not these mechanisms could in principle account for all antibody diversity, there was general recognition at the Congress that in practice they do not. Kabat pointed out how little evidence there is that sequence changes brought about by V-J recombination have any effect on antigen-binding specificity, which depends not only on hypervariable region 3 but on hypervariable regions 1 and 2 as well. He himself, on the basis of an extensive survey of amino acid sequence data, adopts the minority view that the entire V region is constructed from mini-genes separately encoding each of the four framework

regions and three hypervariable regions — a view that has been partly vindicated by the discovery of the separate D and J genes encoding the third hypervariable and fourth framework regions in the heavy chain. But while it is undeniable that, historically, amino-acid sequences have been rather good predictors of otherwise surprising properties of immunoglobulin genes, the balance of opinion is heavily against the mini-gene hypothesis in its extreme form. This is because of the evidence at the DNA level that only one rearrangement takes place between sperm and adult, and that that rearrangement results in transposition of a continuous sequence of DNA containing all three of the first three framework regions with the first two hypervariable regions *in situ*.

The majority view therefore is that any antibody diversity not generated by known recombination mechanisms cannot be explained by recombination at all; and an accumulation of evidence, some of it by no means new, has led to a pronounced switch in emphasis from recombination to somatic mutation as the main generator of antibody diversity.

Ontogeny and diversity

The data that are now recognized as pointing to somatic recombination are

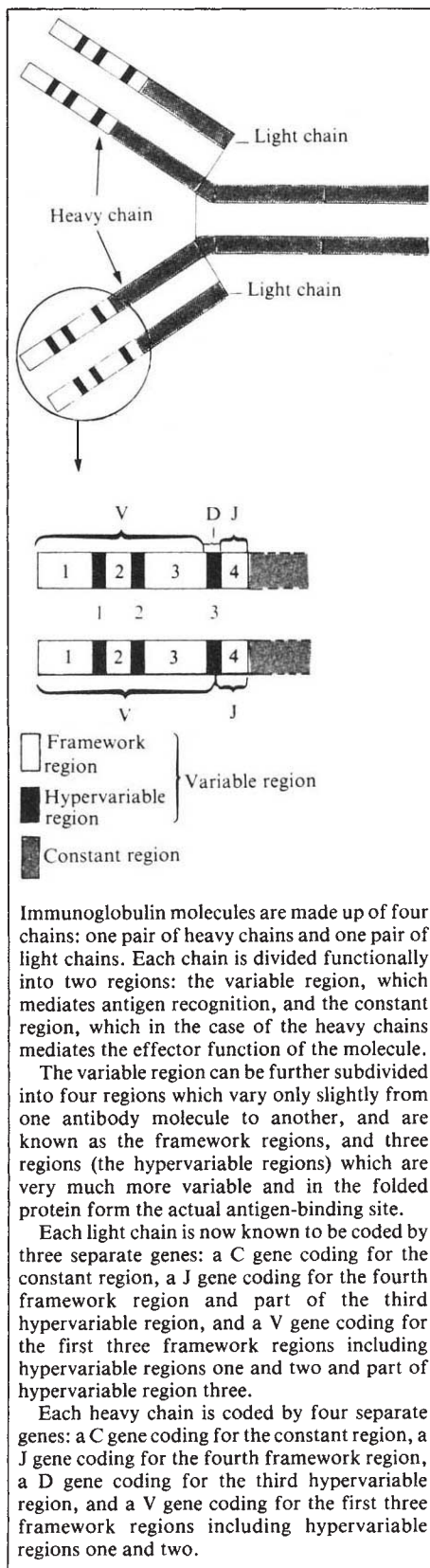
*The 4th International Congress of Immunology was held in Paris on the 21st to 26th July, 1980 and was organized by the Société Française d'Immunologie.

those showing that isolated amino-acid substitutions occur not only in the hypervariable regions but also in the framework regions of secreted immunoglobulin chains. Such substitutions were reported in mouse V_L chains some years ago by Weigert *et al.*⁴, and were in fact apparent from a comparison of the first sequenced germ-line V_L gene with the myeloma gene which had provided the probe¹. A more systematic investigation of departures from germ-line sequences is now under way in a collaboration between K. Rajewsky's laboratory (University of Cologne) and A. Bothwell in Baltimore's laboratory, where 10 germ-line genes have been sequenced for comparison with immunoglobulin genes from a panel of hybridomas secreting antibodies against nitrophenol (NP). This comparison is still in progress, but in the meantime both Rajewsky and P. Gearhart (Carnegie Institute) reported a highly suggestive relationship between immunoglobulin diversity and B cell ontogeny.

This relationship is revealed in a comparison of antibodies of different classes directed against the same antigen. The first class of antibodies to be expressed in an immune response is always IgM, followed by IgG and later perhaps by IgA or IgE, the different classes of antibody being defined by the constant region of the heavy chain, which may be μ , γ , δ , or ϵ . It is believed that the sequential expression of different classes of antibody recognizing the same antigen (the heavy-chain switch) reflects the sequential recombination of a single V-D-J combination with the different C_H genes, by a process known to involve DNA deletion².

Gearhart reported an initial comparison of anti-phosphorycholine (PC) antibodies that suggests, indirectly, that the heavy-chain switch may be associated with somatic mutation in the V region. Partial sequences of 15 hybridoma proteins showed that while all four IgM proteins examined had identical N-terminal sequences, those of eight out of nine IgG proteins had amino-acid substitutions. Seven myeloma IgA anti-PC proteins were also partially sequenced and substitutions were found in 5 of those. Rajewsky has found a similar pattern of substitutions in a preliminary comparison of hybridomas secreting anti-NP IgM and IgG. One important incidental point to emerge from these investigations is that the same idio type was expressed on the different immunoglobulin classes, which invalidates the use of anti-idiotypic antisera for monitoring changes in the V region during B cell ontogeny³.

At present, the evidence in favour of such changes is necessarily indirect, and there is clearly an urgent need for a B cell clone in which a class switch can be induced so that comparison of V regions is possible within a single cell line. (Cell lines that undergo class switches do exist, but they are tumour cell lines of suspect ploidy.)



Immunoglobulin molecules are made up of four chains: one pair of heavy chains and one pair of light chains. Each chain is divided functionally into two regions: the variable region, which mediates antigen recognition, and the constant region, which in the case of the heavy chains mediates the effector function of the molecule.

The variable region can be further subdivided into four regions which vary only slightly from one antibody molecule to another, and are known as the framework regions, and three regions (the hypervariable regions) which are very much more variable and in the folded protein form the actual antigen-binding site.

Each light chain is now known to be coded by three separate genes: a C gene coding for the constant region, a J gene coding for the fourth framework region and part of the third hypervariable region, and a V gene coding for the first three framework regions including hypervariable regions one and two and part of hypervariable region three.

Each heavy chain is coded by four separate genes: a C gene coding for the constant region, a J gene coding for the fourth framework region, a D gene coding for the third hypervariable region, and a V gene coding for the first three framework regions including hypervariable regions one and two.

Ig genes of T cells

While the immunoglobulin molecule steadily yields its secrets to the combined onslaught of restriction enzymes and sequencers, the T cell receptor continues to elude molecular and cellular immunologist alike. This is particularly tantalizing in

view of the evidence at the cellular level that the antigen binding sites of T cell receptors are encoded by immunoglobulin V_H genes. The original evidence for the expression of V_H genes in the T cell receptor was the demonstration that T cell help in antibody production could be induced or inhibited by anti-idiotypic antibodies⁵. Since then both suppressor (T. Tada, Tokyo University; M. Taussig, ARC Babraham; A. Nisonoff, Harvard University) and helper (E. Mozes, Weizmann Institute) cells have been found to bind anti-idiotypic antibodies and to release idiotypic-bearing factors which suppress or induce the production of antibodies to a specific antigen.

Progress on the biochemical characterization of these factors has however been slow, and in the meantime the appealing prospect of unifying T and B cell responses to antigen within a regulatory network based on idiotype determinants of a single repertoire of V_H regions has become fogged with complications. C. Janeway (Yale University), discussing the variable consequences of attempts to manipulate the immune response with anti-idiotypic antibodies, pointed out that there is evidence that T cells have a more restricted repertoire than B cells, since an anti-idiotypic antibody that suppresses only a part of the B cell response to an antigen (the idio type-positive part) can completely eliminate the T cell response to the same antigen. In the light of recent developments, this could simply mean that T cells use the same germ-line V genes but do not subsequently diversify by mutation. However a more serious problem arises in a result reported by H. von Boehmer (Basel Institute of Immunology) who has used an anti- V_H antiserum raised by D. Givol (Weizmann Institute) and his colleagues to stain cytotoxic T cells. To circumvent the problem of antigen specificity and the T cell repertoire, Givol produced an antiserum directed against common V_H framework determinants. This antiserum has been used successfully to isolate T cell factors but von Boehmer finds that it does not stain cytotoxic T cell clones.

Meanwhile, molecular biology laboratories have been competing in the attempt to by-pass the biochemistry altogether and reach for the receptor DNA with heavy-chain probes. Tonegawa, as reported at the Congress by von Boehmer, has used a J_H probe to explore a cloned cytotoxic T cell line and found V genes either in their germ-line arrangement, or rearranged aberrantly, but never rearranged to produce a V-D-J join as in a differentiated B cell.

In spite of the overwhelming evidence that none of the known immunoglobulin C regions is present on T cells, D. J. Kemp (Walter and Eliza Hall Institute of Medical Research) and T. Rabbitts (MRC Laboratory, Cambridge) have used a C region rather than a V region probe and shown, surprisingly, that the gene encoding the μ chain is rearranged both in T lymphoma cells⁶ and in cloned T cells⁷. Kemp's

colleagues have however gone on to show that although the μ gene is transcribed it is not translated⁸.

What, then, is the significance of the apparently futile rearrangement of immunoglobulin genes in T cells? The answer suggested by Kemp is that they reflect the operation in T cells of the same enzymes that mediate the recombination events accompanying differentiation in B cells; and that T cell differentiation, therefore, is probably dependent on the same enzyme system, not necessarily operating on the same genes.

Abortive rearrangements in B cells

Abortive rearrangement of immunoglobulin genes is in fact an extremely common feature of B myeloma cells, in which it was first reported by Lenhard-Schuller *et al.*⁹. It has since been shown that in a large proportion of immunoglobulin-secreting myelomas, rearrangement of both light and heavy chain genes has occurred on both chromosomes, although only one directs the synthesis of immunoglobulin^{10,11}. (The other may however give rise to protein fragments, which have occasionally been reported in biochemical investigations of myeloma cells.)

There are three ways of looking at these rearrangements. One possibility is that they are a peculiarity of myeloma cells and would not be seen in normal B cells if it were only possible to clone one and look at its DNA. This view is increasingly disfavoured. The second, which was emphasized by P. Leder (US National Institutes of Health), is that they may be a part of the price the immune system pays for antibody diversity. For example, one obvious consequence of the variable site of V-J joining is that in some recombined genes the reading frame may be shifted; and detailed investigations at the DNA level have now shown that this indeed accounts for an abortive rearrangement in myeloma MPC 173¹² in which a frame shift resulting from the V-J join on one chromosome produces a stop codon within the J sequence.

Similarly detailed investigations on another myeloma, MPCII, have revealed^{13,14} a rather different kind of abortion, in which the V region on one chromosome recombines with a 'pseudo-joining' sequence, closely homologous to the V-J recombination sequence but falling within the intron between the J regions and the C region. This gives rise to a primary transcript from which the J region is missing and in which the splice signals in the RNA are altered in such a way that the V region sequence is spliced out during RNA processing and the final product of translation is a curious little fragment consisting of a hydrophobic leader sequence attached to the constant region of the κ chain. For abortive rearrangements of this sort however M. Weigert (University of

Philadelphia) does not accept an explanation in terms of necessary sacrifices to the generation of diversity. He adopts the third possible view of abortive rearrangements, which is that they are the mechanism of allelic exclusion¹¹.

One of the numerous problems posed by B cells is that of how they contrive to express the immunoglobulin genes on only one of the two homologous chromosomes of a diploid cell. Once it was realized that immunoglobulin gene expression involves rearrangement of the DNA, an obvious possibility was that the DNA remained in the germ-line configuration on the excluded chromosome. However, restriction digest data on that point were conflicting. The conflict is now resolved: in some myelomas the immunoglobulin genes on the excluded chromosome remain in the germ-line configuration, in others they do not; and furthermore rearrangement cannot be the prerequisite for immunoglobulin gene transcription, since C_L (though never V_L) fragments may be transcribed in some myelomas from an unrearranged chromosome¹¹.

The important point is that in no case has a myeloma cell been shown to express more than one functional chain of each type. Weigert takes the view that this is because immunoglobulin genes have evolved so as to prevent such an occurrence by making functional rearrangements highly improbable. This accounts, he argues, for the survival of the 'pseudo-joining' site described by Leder, and for the conservation of 'pseudo-J' genes, originally described in κ light chains and now for λ light chains and for heavy chains (O. Bernard, Walter and Eliza Hall Institute). 'Pseudo-J' genes are J genes which do not lead to expression after recombination with a V segment. The conservation of such genes in the repertoire of the three different kinds of chain must imply that they have an important function: namely, to ensure that functional rearrangements are so unlikely that they almost never occur on both chromosomes. It follows from this that a large number of B cells probably undergo abortive rearrangements on both chromosomes and die without ever

expressing immunoglobulin.

Further support for this general scheme is to be found in recent evidence on the fate of the κ light chain genes in B cells secreting λ light chains. For B cells have to decide not only which of two allelic sets of λ or κ light chains is to be expressed, but whether they are to be λ or κ in the first place. That the two decisions may be made on a similar basis was first suggested by the discovery⁹ that, just as the genes for the unexpressed allele have been found to be rearranged, so the genes for the unexpressed κ class are rearranged in myeloma cells secreting λ chains. This discovery has now been confirmed and extended by Alt *et al.*¹⁵ who have shown that in two out of four λ -secreting mouse myeloma cell lines, the κ genes are not only abortively rearranged but transcribed and translated into protein fragments. Similarly, Leder and his colleagues have shown that κ genes are rearranged in human B cell lines secreting λ chains.

These data are consistent with the general idea that abortive rearrangements may be the rule rather than the exception. However, preliminary investigations in Leder's laboratory on the converse question of the fate of λ light chain genes in human B cell lines secreting κ chains casts some doubt on whether light-chain class exclusion is really exactly analogous to allelic exclusion within a light-chain class. For in κ -secreting lines, the λ genes seem to remain in the germ-line configuration. The implication of this is that chromosomal rearrangements may take place in a pre-determined order, so that only if recombination of the κ genes fails to produce a functional light chain will the λ genes be rearranged. It is already clear that there is some hierarchy in the rearrangement of genes for different immunoglobulin chains since it is established that heavy-chain genes are expressed before light-chain genes. As soon as a functional light chain is expressed, it can combine with the heavy chain to form a complete cell surface receptor. Alt *et al.* suggest that it is this event that shuts off further rearrangements of immunoglobulin genes so as to exclude the formation of λ chains in cells already expressing κ chains.

According to the evidence so far, then, there is a pecking-order for the DNA-recombining enzyme such that V_H genes are rearranged first, followed by the $V_{L\lambda}$ genes and finally, but only in the absence of existing surface immunoglobulin, the $V_{L\kappa}$ genes.

What is conspicuously missing from this picture is its context. While the ambitions of molecular biologists run to the possibility not only of isolating clones of B cell precursors, but even of dispensing with the cell altogether so as to examine the recombination mechanism without interference, there is on the other hand a real need to understand the events of lymphocyte differentiation in general in the context of the physiological signals that trigger them.

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REVIEW ARTICLE

Insulating ground state
and nonmetal-metal transitions

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For over a decade there has been intense activity in the field of nonmetal-metal transitions in condensed systems in general and in solids in particular. The object of this review is to pinpoint the nature of the insulating ground state and to outline recent trends in our understanding of the metallic state and related phase transition.

ALL condensed systems can be considered as belonging to essentially three canonical states which in their simple limits are: metals, Mott insulators and covalent insulators (P. W. Anderson, personal communication). It is easy to define the metallic state in the simple limit—here the kinetic energy of the electrons dominates all relevant interactions whether between electrons and electrons or between electron and ion cores. The simple metals from the first columns of the Periodic Table especially those of the second, third and fourth columns are well described by this limit (r_s is small, where r_s is electron-electron distance and is a measure of electron-electron repulsion $\sim 1/r_s$ as well as of their kinetic energy $\sim 1/r_s^2$). As the kinetic energy of the electrons (their bandwidth) becomes smaller as we go away from this limit, two distinct types of symptoms usher the transition to the two other canonical states before the system becomes insulating: the characteristic large susceptibility and specific heat of almost magnetic metals such as Pt (ref. 1) which indicate strong repulsive interaction between electrons (henceforth called Mott-Hubbard U)² and on the other hand superconductivity which results from electron-ion interactions (henceforth called λ).

The decreasing bandwidth is achieved in principle by increasing the atom-atom distance which would obviously result if the process is continued until it results in systems of well separated atoms. Individual atoms are in general magnetic when their electrons are in open shells. This limit is represented by the paramagnetic 'Mott' insulators, which include a wide variety of transition metal oxides and sulphides, such as NiO and V_2O_3 ; their magnetic properties show that these cations retain unpaired spins and in spite of their open shells show no metallic conduction. This is because the repulsive energy U needed to put an extra electron on the same orbit is higher than the kinetic

energy (bandwidth W) gained in delocalizing the electron resulting the electron preferring to remain in a localized state on every atom. This is the second canonical limit of $U > W$, which we have designated as 'Mott insulators'. In this limit, the system will permit two classes of excitation: (1) those involving the reversal of a spin in place of the type

$$S_i^+ = C_{i\uparrow}^+ C_{i\downarrow}, \quad S_i^- = C_{i\downarrow}^+ C_{i\uparrow}$$

where S_i are the spin-operators on site i and C s are the creation and annihilation operators. And (2) those involving the actual creation of a free pair of carriers of the form $C_{i\sigma}^+ C_{j\sigma}$, $i-j \rightarrow \infty$. The latter requires the excitation energy U , whereas the former does not (if the spins are not ordered) and for $U \gg W > kT$ we will have a paramagnetic insulator. As long as $W \neq 0$ but remains smaller than U , mixing of the spin-states through electron tunnelling between sites will occur so that the ground state will order antiferromagnetically ($J_{AF} \sim W^2/U$), although we may note that the resulting doubling of the lattice periodicity has nothing to do with the insulating Mott ground state: most Mott insulators remain nonconducting above the Néel temperature. One must point out that the Mott transition to a metallic state at zero temperature is, by definition, a phase transition of first order due to many-electron screening of U (and an electron must necessarily suddenly change from localized to itinerant behaviour) and this has nothing to do with the polemic whether the Hubbard hamiltonian generally used to describe interacting electron system in a narrow band gives a first or a second order phase transition or not.

The third canonical limit of the solids is reached when the electron-ion interaction λ far exceeds the electron bandwidth W . As we approach from the side of a perfect metal ($\lambda = 0$, since by definition free electrons do not see the perturbing potential of the ions), a non-zero λ makes itself felt by phonon-induced resistivity $\sim T^5$ in the low temperature region. In the ground state, however, this non-zero λ has two strikingly related behaviours, both related to λ -induced attractive interaction between two electrons: if $\lambda \ll W$, this results in electron pairing and superconductivity, while for $\lambda \gg W$, we find ourselves in localized pair ground states which are diamagnetic singlet states, the covalent insulators being their extreme canonical limit (their essential diamagnetism differentiates them from the Mott insulators) of this class of solids. It is essential to realize that a purely local one electron-ion interaction results in a non-local two-electron attractive interaction sufficiently strong to overcome the repulsive mutual Coulomb interaction so that paired ground state can occur. The tendency to pairing is quite evident as we look into the Periodic Table. The fifth column semimetals provide an excellent example—Bi, As, Sb. These metals have an outer s-shell completely full leaving three electrons in their p-orbitals, along x , y and z directions. In a tight-binding theory of the cubic structure (all these metals at high pressure are cubic) as developed by Labbé and Friedel³, the dominant hopping

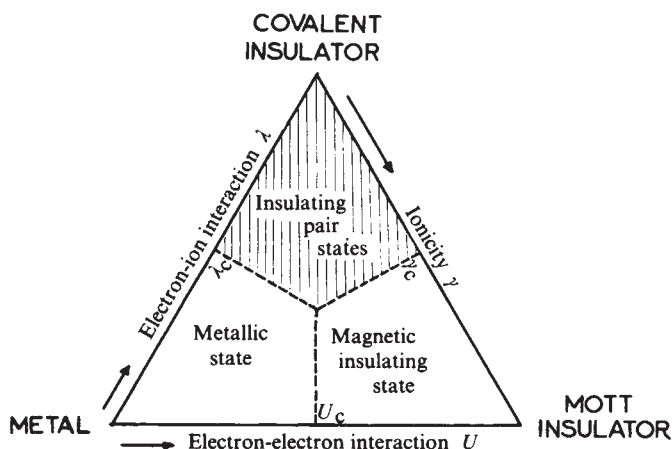


Fig. 1 Three limits of the solid state.

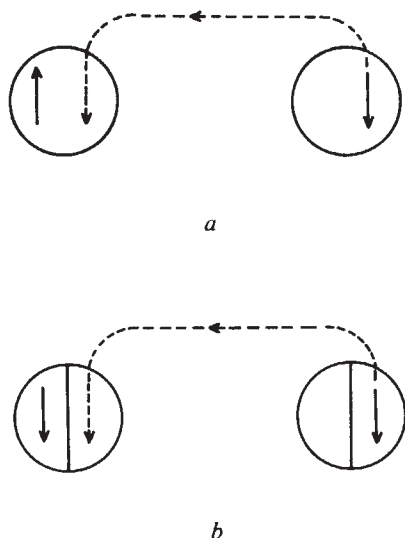


Fig. 2 *a*, Kinetic interaction, antiferromagnetic ground state. *b*, Kinetic interaction, degenerate orbitals, ferromagnetic ground state.

integral is the p_σ -type which gives simply three one-dimensional tight-binding bands each of which will be half filled. The effect of λ would be essentially a lattice distortion, known as 'Peierl's distortion' leading to pairing up of the electrons along x , y and z orbitals and thus one expects a layered structure in which each Bi atom will have three close neighbours in the x , y and z directions, which is what one observes in the actual metals. Note that in these semimetals, the Peierl's distortion leads to a Fermi surface with incomplete gaps, hence very large dielectric constants and small effective mass of the carrier which explain their semimetallic behaviour. The pairing phenomenon leading to Peierl's distortion is a process which competes with superconductivity and if for example Bi or As or Sb can be kept simple cubic, either by pressure or by quenching in impurities, they develop a remarkably high superconducting transition temperature ~ 10 K. Similarly, the A_{15} high T_c transition metal superconductors are equally subject to the martensitic structural instability often before the superconductive transition. This shows that the effects of λ on the metallic ground state are (1) superconductivity, (2) covalent-pairing leading to incomplete gaps at the Fermi surface in some cases—as in the semimetals or uniform distortion as in the A_{15} compounds and (3) for larger λ , the tendency to covalent-pairing becomes so great that we get bipolaronic insulating state⁴ (counterpart of the superconducting ground state) and eventually a covalent insulator like Ge or Si, where one can think of the pseudo-potential interaction λ as having eaten up the whole of the Fermi surface making a complete energy gap all around the Brillouin zone. These three canonical states of the solids—metals, Mott insulators and covalent insulators—are depicted in Fig. 1, in an 'alchemical' triangle (at $T=0$) which shows clearly the metal-nonmetal transition boundary to be expected as we move away on U -axis or λ -axis from the simple metal limit. The third axis that ties up the covalent insulator with the Mott insulator is the ionicity parameter γ (Pauling-Phillips-Van Vechten⁵) or the Madelung energy contribution that increases as we approach the Mott-insulation limit. On the Mott insulator side, anions are completely diamagnetic (unlike the cations), filling up the closed rare-gas shells. Approaching the boundary towards covalency, the outermost anionic wave functions spread out into the cationic orbitals. On the other side of the covalent ionic boundary as the ionicity decreases, we start building up the pair states like H_2 , O_2 or N_2 or larger groups like benzene, where internally within the molecule we have mobile pair of electrons, but overall a molecular insulating crystal. For the rest of the article, we shall not elaborate any further on this third axis of the alchemical triangle.

Mott insulators

The transition from a metallic to Mott-insulating ground state is by definition accompanied by no change of lattice symmetry. The states on either side of the transition remain paramagnetic (from Pauli to Curie-Weiss behaviour) and is brought about as the lattice parameters are expanded such that diminution of W leads to U/W exceeding some critical value ~ 1 . A rough idea of this critical value can be obtained from elementary arguments. Assume that U is small and the system is in the metallic state with one electron per atom. If we now remove one electron from its site and let it travel around the system, it will on each site have an energy $\epsilon_0 + U$ due to repulsive interaction of the electron already sitting there. If the electron on its tour through the crystal happens to catch the hole it left, it will have an energy ϵ_0 at the side where it happens. This means the electron will recognize the hole as a potential well of depth U (and radius R). We can easily find out when these two body scattering events start to form a bound state. This is an elementary problem in quantum mechanics and gives us the binding condition as

$$\frac{8}{\pi^2} \frac{m^* U}{h^2} R^2 > 1$$

where m^* is the effective mass of the electron. Fitting the density of states to a rectangular density of states function and a bandwidth W , we can rewrite the bound-state condition as

$$\frac{U}{W} > \frac{\pi}{4} \left(\frac{4\pi}{9} \right)^{1/3} \sim 0.88$$

which is not very different from the Hubbard solution.

Are there any experimental systems that show the Mott ground state? Unfortunately the paramagnetic insulating ground state at $T=0$ is not the lowest energy state due to exchange effect between the electrons and is thus unobservable. This exchange effect can be essentially of two kinds: if the orbitals are non-degenerate and we have one electron per atom, the exchange is a so-called kinetic exchange and is antiferromagnetic, as seen in Fig. 2*a*. The hopping of electron from one site to another is only possible if they have opposite spins; (for identical spins, the Pauli principle prevents all electron transfer) so that the lowest energy state is antiferromagnetic, with a $J_{AF} \sim W^2/U$. Thus in the ground state, we will find an insulating and antiferromagnetic material. On the other hand, suppose the lowest energy orbitals are degenerate (say doubly degenerate) and we have one electron per atom. We expect the system to be metallic as the band will be $1/4$ full but it will be a ferromagnetic

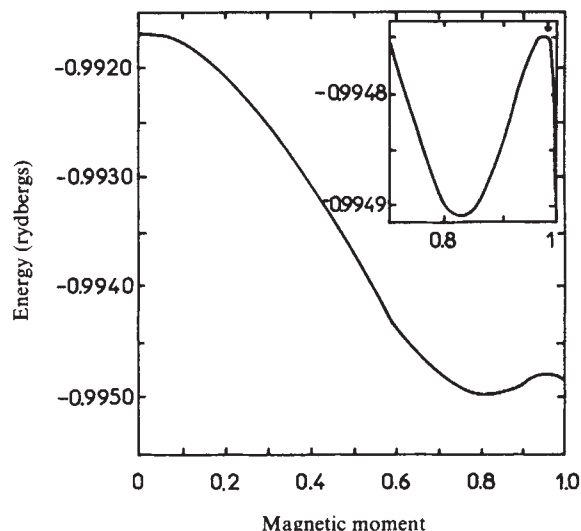


Fig. 3 Total energy at the first order metal-insulator transition as a function of the magnetic moment in the unit cell at $r_s = 2.84$. The inset shows an enlargement of the double-well structure. The arrow indicates that value of M for which the bottom of the upper spin band crosses the top of the lower spin band.

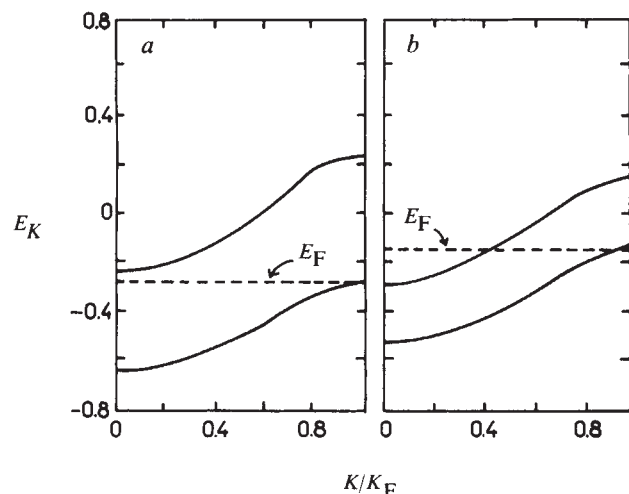


Fig. 4 Spin-split bands at the first order phase transition. *a*, The bands and the existence of an energy gap when the electrons are completely spin aligned ($M = 1$). *b*, The overlapping bands which occur at the second energy minimum (see Fig. 3) for $M = 0.82$.

metal due to the following simple argument⁶ (Fig. 2*b*). If the two electrons on the adjacent sites have identical spins but on different orbitals, they can profit from Hund's rule coupling when one hops, as shown, to give

$$J_{\text{ferro}} \sim -\frac{W^2}{U - J_0}$$

where J_0 is Hund's rule coupling energy, while

$$J_{\text{AF}} \sim \frac{W^2}{U}$$

These two behaviours are very nicely illustrated in the transition metal dichalcogenide crystals of pyrite structure as we go from CoSe_2 to NiS_2 . In CoSe_2 , the doubly degenerate e_g orbitals has one electron per atom; CoSe_2 is metallic and ferromagnetic. In NiS_2 , the same e_g orbitals now have to accommodate two electrons per atom; NiS_2 is insulating and antiferromagnetic. Thus NiS_2 (refs 7, 8) is a very good candidate for a Mott insulator. And under pressure at ~ 30 kbar, NiS_2 does go to a metallic ground state. A pure Mott transition has also been claimed⁹ for V_2O_3 and its Cr-doped alloys, at its high-temperature transition to the insulating paramagnetic state. This is one of the most completely studied system; controversy remains, however, because the paramagnetic insulating state is reached as temperature is increased and the antiferromagnetic insulating ground state is accompanied by lattice symmetry change considerably complicating the implication of correlation 'only' in this system.

Recently, more quantitative calculation of the Mott-Hubbard transition had been performed by Rose *et al.*¹⁰ For the system of one electron per atom, they have obtained as a function of r_s the ground-state energy (Fig. 3) energy dispersion (Fig. 4*a, b*) as well as magnetic moment per site (Fig. 5). We see very nicely the first-order metal-nonmetal transition at an $r_s = 2.84$ predicted by Mott (this corresponds to Mott's universality criterion of $n^{1/3} a_0 = 0.22$). The double-well structure of Fig. 3 is the signature of the first-order phase transition. Considering Fig. 4*a* drawn for $r_s = 2.84$, we see the band occupation when magnetization per site $M = 1$, that is, complete spin polarization of the system. Consider taking a few electrons out of the lower band and putting them in the upper band. We must pay an energy ΔE to do this as Fig. 3 shows. However, there is a second effect which causes this energy increase to stop and leads to the formation of the second minimum at $M = 0.82$. This effect results from the fact that the electrons and holes created by the transfer interact and reduce the energy gap between the two bands. In fact this effect is sufficiently large so that when we put enough electrons

in the upper band, the bottom of the upper band crosses the Fermi surface of the lower band and energy is gained by transferring electrons to the upper band. The result is the minimum at $M = 0.82$ (Fig. 4*b*). The qualitative behaviour is exactly as described by Mott's original arguments for the first order nature of the metal-insulator transition. These calculations also show formation of a spin moment in a second order phase transition at $r_s = 2.74$ (Fig. 5).

To conclude this section, we will mention a completely different class of systems that show metal-nonmetal transition and are probably closest to the spirit of the original idea of Mott—that as the atom-atom distance is decreased, the system must go suddenly to the nonmetallic state. These systems are the low-density fluids or dense metallic vapours at high critical temperatures or pressures, and have been studied extensively by Hensel and co-workers¹¹ over the last decade. The most thoroughly studied is mercury (critical temperature $1,760^\circ\text{C}$, critical pressure $1,510$ bar for liquid-vapour transition) and it shows a transition to a fluid nonmetal as the pressure is varied. The fluid alkali metals on the other hand like Cs or Rb (ref. 12) show Mott transition at their critical points of gas-liquid transition with anomalous increase of the magnetic susceptibility as the Mott localization limit is approached (for Cs this occurs at around $1,800^\circ\text{C}$).

Bipolaronic insulators

In complete contrast to the Mott insulators, the bipolaronic insulators are characterized as a ground state of localized pairs of electrons in singlet state. Such an insulator is thus completely diamagnetic and is obtained as we go along the λ axis of Fig. 1, until a critical λ_c is reached at which the Fermi surface is unstable to the formation of these pair states. Unlike Mott insulators, the bipolaronic insulators are actually seen experimentally as a ground state¹³ in a wide variety of solids and have opened up quite a new field of investigations.

The idea behind the formation of a bipolaron is really quite simple: in order for two electrons otherwise itinerant in an empty band to come together (either on the same site or on two nearby sites) and stay together localized, the lattice must locally deform to couple these two electrons together through a deformation-induced attractive interaction $\sim g_0^2/M\omega_0^2$ (where g_0 is an electron-lattice interaction term, M the atomic mass, ω_0 a lattice frequency) so as to compensate and exceed the instantaneous Coulomb repulsion $v(\sim e^2/\epsilon r_0)$ of the underformed lattice. The total energy of the two-electron system is then

$$E_T = \frac{g_0^2}{M\omega_0^2} - v$$

When E_T exceeds $W/2$, the two electrons will emerge from the bottom of the band as a localized bound pair state. This situation is schematically shown in Fig. 6 where E_T is plotted against

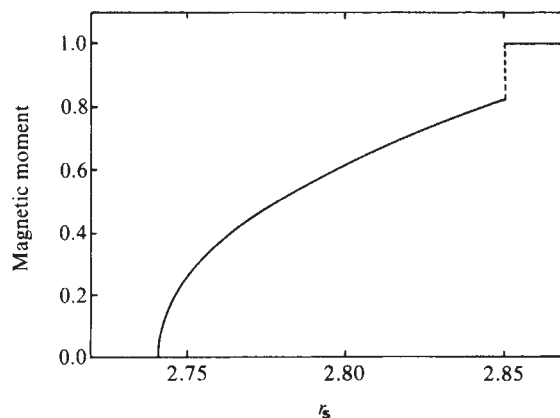


Fig. 5 The magnetic moment in the unit cell as a function of r_s . The behaviour between $r_s = 2.74$ and 2.84 is square-root-like. At $r_s = 2.84$, there is a discontinuous change to the fully spin-aligned state.

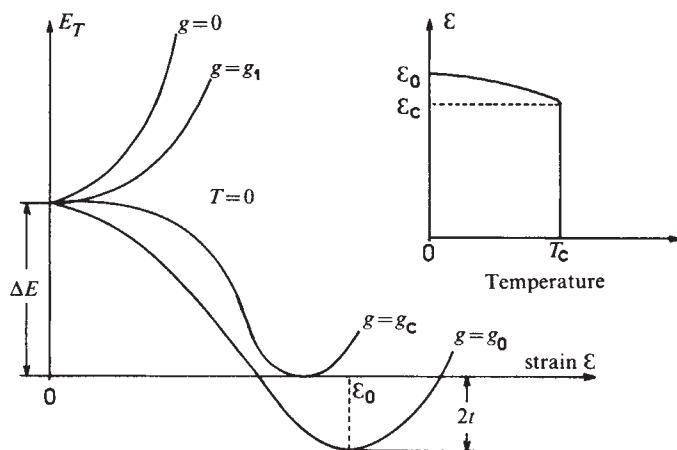


Fig. 6 Total energy of electron pair as a function of lattice deformation.

lattice deformation ε for different values of the coupling constant g_0 and we observe the critical g_0 at which the bound state for the two electrons appears. The transition to the insulating state is sudden and as we raise the temperature, the appearance of the metallic state is characterized by the vanishing of the deformation (insert Fig. 3). Such a pair is called a bipolaron in analogy with polarons. Note, however, that unlike polaronic concepts, bipolaron formation is not necessarily due to optical phonons. Ti_4O_7 (ref. 13) and its alloys remain the most extensively studied bipolaronic insulators. There are several important points that need however to be emphasized:

- (1) In nature, it is rare¹⁴ to find a $s = 1/2$ system that does not have a diamagnetic ground state.
- (2) The interaction between the two electrons is really between two strain centres (two 'polarons'), that is, elastic interaction and can be long-range, highly anisotropic and oscillating in character.
- (3) The bipolarons stand exactly in the same relationship to Bardeen-Cooper-Schrieffer superconducting pairs as does a localized electron to an itinerant one. A phase diagram between superconducting ground state and an insulating

bipolaronic ground state has recently been suggested¹⁵ as a function of the coupling strength λ .

(4) Formation of bipolarons is to be distinguished from the Peierl's transition¹⁶; unlike the latter, there is no lattice symmetry change involved in the former. In this sense, the bipolarons also differ from the insulating ground states of the so-called layer compounds due to charge density wave formation where the lattice periodicity is modulated^{7,8}.

We have attempted a synthesis between what we called the three simple canonical states of solids—metals, Mott insulators and covalent insulators. No attempt has been made to include all varieties of metal-nonmetal transition—for example the valence transitions¹⁷ in rare-earth compounds, or the transitions in lower dimensionality¹⁸ solids or Anderson transitions^{19,20} in disordered systems. Each of these are in a rapidly developing state both theoretically and experimentally and it is quite impossible to include any of them in this short review. The reader is referred to specific articles dealing with these topics. *Note added in proof:* A complete calculation of the Goodenough-Kanamori rules and their relevance to Mott insulators has recently been published²¹.

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ARTICLES

Improved conversion of methanol to single-cell protein by *Methylophilus methylotrophus*

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The glutamate dehydrogenase gene of Escherichia coli has been cloned into broad host-range plasmids and can complement glutamate synthase mutants of Methylophilus methylotrophus. Assimilation of ammonia via glutamate dehydrogenase is more energy-efficient than via glutamate synthase, thus the recombinant organism converts more growth substrate, methanol, into cellular carbon.

A CRITICAL factor in determining the suitability of a micro-organism for use in the production of single-cell protein (SCP) is the efficiency with which it converts substrate carbon into cellular carbon¹. A major reason why the obligate methylotroph

Methylophilus methylotrophus was selected for use in the ICI process to produce SCP from methanol was its high 'carbon conversion efficiency'. However, we have identified a potential source of methanol wastage due to its mode of ammonia assimilation. Instead of using glutamate dehydrogenase (GDH) to catalyse the conversion of ammonia and 2-oxoglutarate into glutamate, this organism uses a two-stage pathway, dependent

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on glutamine synthetase (GS) and glutamate synthase (GOGAT), to carry out the same function (Fig. 1)². The latter pathway requires one extra molecule of ATP per molecule of glutamate formed.

M. methylotrophus probably uses the energetically suboptimal pathway for ammonia assimilation because of the environment in which it evolved. GS has a much higher affinity for ammonia than GDH³ and is therefore more effective in scavenging for low concentrations of ammonia. Since ammonia concentrations are not growth-rate limiting during SCP production, exchanging the ammonia assimilation pathway used by *M. methylotrophus* should result in the organism consuming less energy for this process; correspondingly more methanol should be available for protein synthesis. We have used recombinant DNA techniques to carry out this exchange by introducing the *Escherichia coli* *gdh* gene, which codes for GDH activity, into a mutant of *M. methylotrophus* which lacks GOGAT.

Cloning the *E. coli* *gdh* gene

The best available strain for screening for a cloned *E. coli* *gdh* gene was *E. coli* CB100, which is deficient in both GDH (*gdh*⁻) and GOGAT (*gltB*⁻) and therefore requires glutamate for growth⁴. Derivatives which have received a *gdh* gene from another source should be readily selectable by virtue of their glutamate independence. However, because CB100 is λ -resistant and a relatively poor transformation host we initially cloned the *gdh* gene by *in vivo* techniques using RP4::Mu (refs 5, 6).

A *gltB*⁻ *E. coli* strain (obtained from Dr J. Cole) was lysogenized with Mu *cts* and a derivative of RP4 carrying Mu *cts* was transferred into it. This strain was grown overnight at 37 °C and was then mated non-selectively at 30 °C with a derivative of CB100 which is *recA*, *rif*^r and which is lysogenic for Mu *cts* (CL412). Progeny of this mating were transferred to plates of minimal medium deficient in glutamate but containing rifampicin and kanamycin. This should select for progeny of CL412 which have received plasmid RP4 derivatives carrying a segment of the donor strain's chromosome containing a functional *gdh* gene (that is, an RP4'*gdh* plasmid). Several methods were used to prove that some of the progeny of this mating and selection did carry authentic RP4'*gdh* plasmids. First, the plasmids could be transferred through an intermediary host (C600 *recA*) and back into CL412, selecting only for kanamycin resistance, and they still conferred glutamate independence on the latter strain. Second, elimination of the RP4-derived plasmid from CL412 by mating in an incompatible, trimethoprim-resistant (*Tp*^r), P-group plasmid, R751 (ref. 7), resulted in simultaneous loss of glutamate independence. Finally, enzyme assays showed that

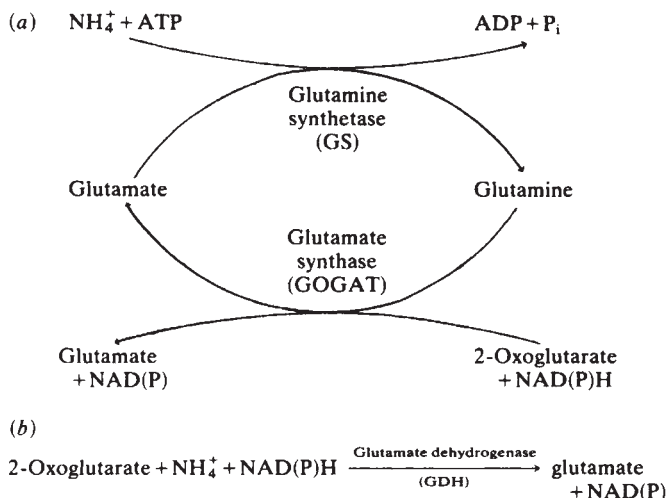


Fig. 1 Pathways of ammonia assimilation by bacteria. a, GS/GOGAT; b, GDH.

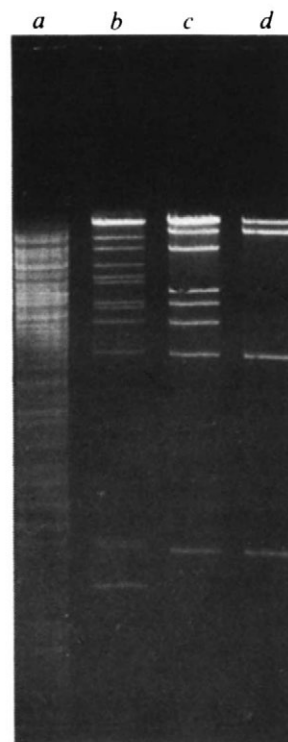


Fig. 2 Comparative *Pst*I restriction endonuclease analysis of RP4' *gdh* DNAs with RP4 and *E. coli* DNAs. RP4' *gdh* plasmid DNAs were prepared from fresh 100 ml overnight cultures of CL412 cells by a Triton X-100 (0.05%)/lysozyme lysis procedure¹⁴, followed by 2 min vigorous vortex mixing and by centrifugation with ethidium bromide and caesium chloride¹⁵, without a preliminary clearing spin. The closed circular DNA was collected dropwise, after preliminary aspiration of the dense linear DNA band, and was then dialysed for 2–3 h against 0.01 M Tris (pH 7.5); 1 mM EDTA (TE buffer) before phenol extraction, *n*-butanol extraction, ethanol precipitation and re-dissolving in TE buffer. RP4 DNA was prepared in essentially the same way and was a gift from G. S. Sharpe. *E. coli* C600 DNA was prepared as described by Marmur¹⁶, except that the cells were lysed with lysozyme and Triton X-100. DNAs were digested with *Pst*I (MRE Porton) in 30 μ l of 20 mM Tris (pH 7.5), 10 mM MgCl₂, 50 mM (NH₄)₂SO₄ and 100 μ g ml⁻¹ BSA for 60 min at 30 °C, before incubation at 70 °C for 10 min and electrophoresis on a 40 cm $\times$ 0.3 cm 1% agarose gel at 200 V overnight, under the conditions of Sharp *et al.*¹⁷ The gel was stained in 2 μ g ml⁻¹ ethidium bromide for 30 min, washed in water for 30 min and photographed under UV light. a, *E. coli* C600; b, RP4' *gdh*3; c, RP4' *gdh*4; d, RP4.

when glutamate independence was conferred by these plasmids it led to the acquisition of GDH and not GOGAT activity.

*Pst*I cleavage of plasmid DNA prepared from some of the RP4' *gdh* carrying strains gave the gel electrophoretic patterns shown in Fig. 2. Although fragments common with RP4 are clearly present, the RP4' *gdh* plasmids are much larger and give more complex restriction patterns than RP4 itself. Similarly, the plasmids RP4' *gdh* .3 and RP4' *gdh* .4 have homologous *Pst*I DNA fragments which they do not share with RP4. However, the RP4' plasmids clearly differ from each other, RP4' *gdh* .3 being substantially larger than RP4' *gdh* .4. All of these features might be anticipated because of the method of construction of the plasmids, which should have resulted in the transfer of substantial, but variably sized pieces of the *E. coli* chromosome to RP4, together with two copies of the Mu *cts* phage genome⁶. Following transfer of these RP4' *gdh* plasmids directly from *E. coli* to *M. methylotrophus*, by selection for antibiotic resistances conferred by RP4, we were unable to detect GDH activity; this could be due to the probable instability of plasmids of this structure in a recombination-proficient host⁶, or to inefficient transcription of the heterologous DNA.

To isolate a more defined DNA fragment carrying the *E. coli* *gdh* gene, we cloned DNA from three of the RP4/*gdh* plasmids on to the vector plasmid pACYC184 (ref. 8), using the restriction enzymes *Hind*III, *Bam*H1 and *Sal*I. Only with the latter enzyme were substantial numbers of recombinant plasmids obtained which caused CB100 to become chloramphenicol-resistant and glutamate-independent. Plasmid DNA, isolated from four such transformants, contained in each case, a 7.6-kilobase fragment of *E. coli* genomic DNA, flanked by *Sal*I targets (Fig. 3). Enzyme analyses on CB100 strains carrying these pACYC184 derivatives, which confer glutamate independence, show convincingly that this independence is caused by the presence of high levels of GDH (Table 1a). The enzyme level apparently reflects the multi-copy nature of the pACYC184 vector plasmid, which indicates that increased gene dosage can cause enhanced GDH production in *E. coli*. Minicell experiments have confirmed that the principal new polypeptide, resulting from the presence of the 7.6-kilobase DNA fragment in the pACYC184 recombinant plasmids, co-migrates with purified *E. coli* GDH on SDS/polyacrylamide gels and is immunoprecipitable with anti-*E. coli* glutamate dehydrogenase antibody (T. C. Hodgman, personal communication). We are therefore confident that the *Sal*I-derived pACYC184 recombinant plasmids we have isolated carry a functional *E. coli* *gdh* gene and have designated them pACYC184/*gdh* plasmids.

Construction of plasmids to transfer the *E. coli* *gdh* gene to *M. methylotrophus*

Because pACYC184 is not transferable to *M. methylotrophus*, other plasmid vectors have had to be used to achieve *gdh* gene transfer; these are shown in Fig. 4. pRP301 is a low copy-number conjugative derivative of RP4 which has lost one of the two *Sal*I sites of that plasmid, together with kanamycin resistance (*Km*^r; ref. 9). Like RP4 it can transfer itself from *E. coli* to *M. methylotrophus* and maintain itself in its new host. pTB70 is a derivative of the multi-copy plasmid R300B (ref. 9), which confers streptomycin resistance (*Sm*^r) and sulphonamide resistance (*Su*^r) and into which the *Km*^r transposon Tn5, together with a small additional piece of DNA, has been inserted. Tn5 gives pTB70 a single *Sal*I site¹⁰. Like R300B, pTB70 can be mobilized by RP4 to *M. methylotrophus* and is capable of stable maintenance in this organism.

The *E. coli* *Sal*I *gdh* fragment was readily transferred from pACYC184/*gdh* to the pRP301 and pTB70 vectors. Donor and recipient plasmid DNAs were mixed, cleaved with *Sal*I, ligated and transformed into CB100, selecting for ampicillin-resistant (*Ap*^r) or *Km*^r transformants respectively and screening these for glutamate independence. Representative examples of the pRP301/*gdh* and pTB70/*gdh* recombinant plasmids isolated from such glutamate-independent transformants are shown in Fig. 3. Enzyme activities in CB100 cultures carrying such recombinants are consistent with the gene dosage effects noted above (Table 1a); pTB70/*gdh* plasmids in particular are of interest as they give rise to the highest levels of GDH in *E. coli* of any plasmids we have constructed so far.

pRP301/*gdh* recombinant plasmids could be transferred directly into *M. methylotrophus* from CB100 by selection for *Ap*^r. However, unlike RP4, neither pRP301 nor pRP301/*gdh* was found to be stable in *M. methylotrophus*. After growth for 11 generations on ampicillin-free medium, only about 50% of the cells remained resistant to this antibiotic. This phenomenon was not observed with *E. coli* cultures carrying the equivalent plasmid. Nevertheless ampicillin selection was routinely maintained for all strains carrying them. pTB70/*gdh* plasmids can efficiently be mobilized into *M. methylotrophus* from *E. coli* by RP4, selecting for *Sm*^r transconjugants. Some of these transconjugants stably acquired both RP4 and pTB70/*gdh*, others only acquired the latter plasmid, as judged by their antibiotic resistances. Plasmid DNA isolated from *M. methylotrophus* carrying either pRP301/*gdh* or pTB70/*gdh* was indistinguishable from the corresponding plasmid DNAs isolated from *E. coli* strains,

as judged by restriction endonuclease analysis. To determine whether the *E. coli* *gdh* gene which had been transferred to *M. methylotrophus* on pRP301/*gdh* and pTB70/*gdh* plasmids gave rise to functional GDH, enzyme assays were performed on cultures which had been grown in the presence of 100 µg ml⁻¹ ampicillin or 15 µg ml⁻¹ streptomycin, respectively, to ensure retention of the plasmids in the absence of any means of directly selecting for *gdh* gene retention. As shown in Table 1b, *M. methylotrophus* cultures carrying either pRP301/*gdh* or pTB70/*gdh* plasmids produce significant levels of GDH as well as normal levels of GOGAT. *M. methylotrophus* itself produces only GOGAT. The *E. coli* *gdh* gene therefore appears to be functional in *M. methylotrophus* without further manipulation, although the efficiency of the expression is substantially reduced. The impact of gene dosage is shown in that *M. methylotrophus* carrying pRP301/*gdh* contains substantially lower levels of GDH than when carrying the multi-copy plasmid pTB70/*gdh* (Table 1b). The level of GDH in *M. methylotrophus* pTB70/*gdh* cultures is about 45% of that present in *gdh*⁺ *E. coli* strains and this might be expected to be sufficient to allow efficient growth of a glutamate synthase-deficient *M. methylotrophus* strain.

Table 1 GDH and GOGAT activities in strains of *E. coli* and *M. methylotrophus*

(a) <i>E. coli</i> strains	Cofactor:	Enzyme	
		GDH NADPH	GOGAT NADPH
C600		1010	215
CB100		ND	ND
CB100 (pACYC184/ <i>gdh</i>)		4880	ND
CB100(pRP301/ <i>gdh</i>)		1390	ND
CB100(pTB70/ <i>gdh</i>)		6130	ND
(b) <i>M. methylotrophus</i> strains	Cofactor:	NADPH	NADH
<i>M. methylotrophus</i>	30	ND	78
<i>M. methylotrophus</i> (pRP301/ <i>gdh</i>)	30	75	72
<i>M. methylotrophus</i> (pTB70/ <i>gdh</i>)	30	453	75
<i>M. methylotrophus</i>	37	ND	229
<i>M. methylotrophus</i> (pTB70/ <i>gdh</i>)	37	835	115
<i>M. methylotrophus</i> 56.2 (pTB70/ <i>gdh</i>)	37	757	ND

E. coli strains used in enzyme assays were grown in M9 medium²² supplemented with appropriate amino acids at 40 µg ml⁻¹ and thiamine at 4 µg ml⁻¹. *M. methylotrophus* strains were grown on medium containing (per litre): (NH₄)₂SO₄, 1.8 g; MgSO₄·7H₂O, 0.2 g; NaH₂PO₄·2H₂O, 1.4 g; K₂HPO₄, 1.9 g; FeCl₃, 0.98 mg; CuSO₄·5H₂O, 0.02 mg; MnSO₄·4H₂O, 0.1 mg; ZnSO₄·7H₂O, 0.1 mg; CaCO₃, 1.8 mg. pH was adjusted to 6.85 and methanol added to give a final concentration of 0.5% v/v. Aliquots (500 ml) of pre-warmed media were inoculated with 50 ml overnight cultures grown in the presence of 100 µg ml⁻¹ chloramphenicol, 100 µg ml⁻¹ ampicillin or 15 µg ml⁻¹ streptomycin, where appropriate, to ensure selection for plasmid retention. The cultures were grown with shaking aeration at 37°C to an A₆₅₀ of 0.5, harvested by centrifugation, washed with 10 ml ice-cold buffer (22 mM KH₂PO₄; 50 mM Na₂HPO₄; 85 mM NaCl and 0.1 mM MgSO₄) and stored overnight on ice as cell pellets. Cell extracts were prepared by resuspending the pellets in 1 ml buffer, sonication and pelleting cell debris by centrifugation at 80,000 g for 60 min. Enzyme assays were performed at 30°C, unless otherwise indicated. The assay mix contained (in a 3 ml cuvette): 50 mM HEPES buffer pH 7.5; 1 mM EDTA; 1 mM 2-oxoglutarate; 0.16 mM NAD(P)H; cell extract 0.01–0.1 ml. The reaction was initiated by the addition of substrate to give a final concentration of 10 mM, using NH₄Cl to assay GDH or L-glutamine to assay glutamate synthase. The oxidation of NAD(P)H was followed at 340 nm using a Perkin-Elmer 554 spectrophotometer and a water-jacketed cuvette holder. Enzyme activities are given in nmoles NADH or NADPH oxidized min⁻¹ mg protein⁻¹, protein concentration being determined by the method of Lowry *et al.*²³. ND = No detectable activity.

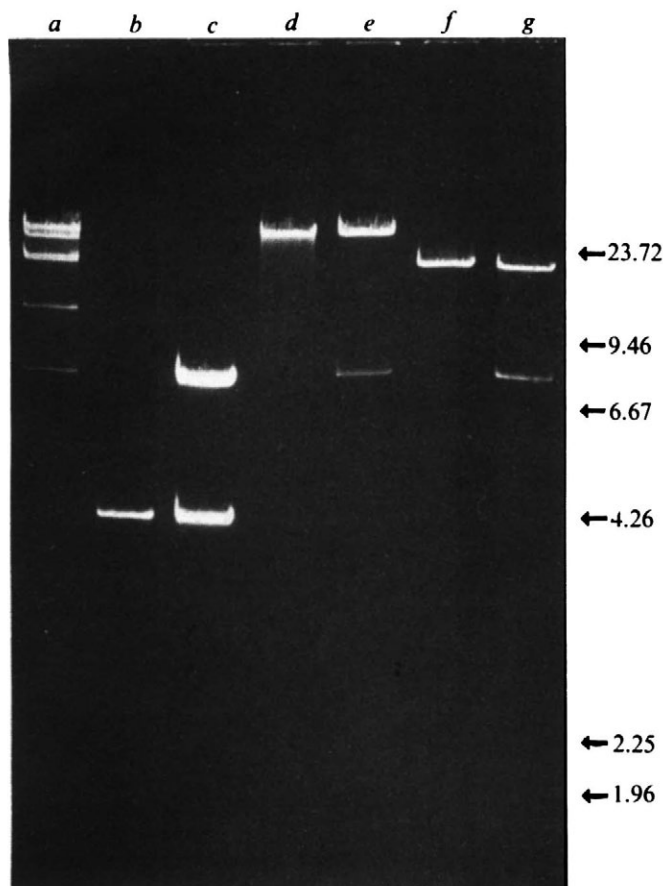


Fig. 3 Comparative *SalI* restriction endonuclease analysis of RP4/*gdh*4, pACYC184/*gdh*, pRP301/*gdh* and pTB70/*gdh* plasmids. Plasmid DNAs were prepared from suitable CB100 derivatives as described in the legend to Fig. 2. *SalI* digests were performed in 30 μ l volumes with enzyme obtained from Bethesda Research Laboratories, under the recommended conditions. Electrophoresis, staining and photography were also as described in Fig. 2. a, RP4/*gdh*4; b, pACYC184; c, pACYC184/*gdh*; d, pRP301; e, pRP301/*gdh*; f, pTB70; g, pTB70/*gdh*. The size markers were obtained by running a *HindIII* digest of λ DNA in a parallel track, and are given in kilobases.

Isolation and characterization of GOGAT deficient mutants of *M. methylotrophus*

To ensure that *M. methylotrophus* carrying the cloned *E. coli* *gdh* gene was entirely dependent upon the GDH pathway for ammonia assimilation, it was necessary to block the existing GS/GOGAT pathway. Elimination of GS activity would result in a glutamine requirement which would not be supplemented by the presence of GDH activity; it was therefore necessary to block the GS/GOGAT pathway by isolating a mutant deficient in GOGAT.

Since GOGAT functions in the biosynthesis of glutamate (Fig. 1), through which most ammonia is assimilated into the cell, the obvious phenotype of mutants which have lost this activity would be an ability to grow on glutamate but not ammonia as sole nitrogen source². However, *M. methylotrophus* cannot use glutamate as sole nitrogen source; uptake experiments suggest that this is due to impermeability to this compound (J. McNairney, personal communication). Permeability mutants which are able to use glutamate as a nitrogen source can be readily selected from *E. coli* W (ref. 11), which resembles *M. methylotrophus* in poor uptake of glutamate. However, we have been unable to isolate such mutants from the latter organism.

A search was initiated to find alternative nitrogen sources (Table 2), but of the compounds tested only glutamine gave a

positive result. However, we have been unable to isolate either glutamine auxotrophs of *M. methylotrophus* or alternatively mutants able to grow on glutamine but not ammonia as sole nitrogen source. Furthermore, experiments with radiolabelled glutamine indicated that it too cannot be taken up by the cell (W. Naylor, personal communication). Since *M. methylotrophus* can grow on extremely low concentrations of ammonia, and as glutamine is rather unstable, it is conceivable that apparent growth on glutamine is due to the presence of ammonium ion impurities. Alternatively, *M. methylotrophus* may have some mechanism for breaking down glutamine extracellularly and growing on the ammonia thus produced.

These results indicated that a mutant which had lost GOGAT activity would not be able to grow on any of the nitrogen sources tested. A strategy was therefore adopted of isolating temperature-sensitive mutants and screening these for strains able to grow at the non-permissive temperature following the introduction of pTB70/*gdh* into them. Out of a total of 550 mutants, isolated after mutagenesis with *N*-methyl-*N*¹-nitro-*N*-nitroso-guanidine, four were found which could grow at the non-permissive temperature (37 °C) when they had received this plasmid. However, these mutants failed to grow at 37 °C when pTB70 was transferred into them, indicating that they were complemented by a function encoded by the 7.6-kilobase pair cloned fragment of *E. coli* DNA carried by pTB70/*gdh*. Similarly, when the mutants carried the low copy-number plasmid pRP301/*gdh*, which only gives rise to low levels of GDH in *M. methylotrophus* (Table 1b), they grew poorly at 37 °C. Mutants carrying pRP301 alone, failed to grow at this temperature.

Mutants of *E. coli* lacking GOGAT activity and relying on GDH for growth on ammonia are unable to grow on media supplemented with 0.075 mM NH_4^+ , although the wild-type organism will grow slowly on such media². This is probably due to the difference in the affinity for ammonia of the enzymes involved³. We therefore tested the ability of the putative temperature-sensitive GOGAT mutants of *M. methylotrophus* carrying pTB70/*gdh* to grow on plates containing 0.07 mM NH_4^+ at 30 °C and 37 °C. The results for one such mutant, 56.2, are shown in Table 3. Whereas, at 30 °C, both the wild-type and the temperature-sensitive mutants formed colonies on plates with ammonia concentrations down to 0.07 mM, at 37 °C the temperature-sensitive mutants complemented by pTB70/*gdh* would not grow on 0.07 mM ammonia. The latter strains grew well, however, on both 1 mM and 15 mM ammonia. This strongly suggests that these strains use GDH for growth and are

Table 2 Nitrogen sources for *M. methylotrophus*

Compound	Growth
NH_4^+	+
L-glutamine	+
L-glutamate	—
L-aspartate	—
L-proline	—
L-arginine	—
L-alanine	—
γ -L-glutamyl-L-glutamate	—
L-alanyl-L-glutamate	—
L-pyroglutamate	—
N-acetyl-L-glutamate	—
DL- α -methyl glutamate	—
L-glutamic acid dimethyl ester	—
L-glutamic acid diethyl ester	—
γ -benzyl-L-glutamate	—

Overnight cultures of *M. methylotrophus* were centrifuged, washed in buffer and inoculated into liquid medium of the composition described in the legend to Table 1, except that the compounds to be tested were the sole nitrogen source and were assessed at both 5 mM and 10 mM. Growth was scored after 48 h incubation at 37 °C. + Indicates growth, — indicates no growth.

unable to use GOGAT for ammonia assimilation at 37 °C. Finally, enzyme assays demonstrated that temperature-sensitive mutants complemented by pTB 70/*gdh* and grown at 37 °C lack detectable GOGAT activity but have high levels of GDH synthesized from the plasmid-carried *gdh* gene derived from *E. coli*. The results for mutant 56.2 are shown in Table 1b.

Thus *M. methylotrophus* has now been genetically modified so that it can assimilate ammonia via the energetically more favourable GDH route. The resultant organism *M. methylotrophus* 56.2 carrying pTB70/*gdh*, was tested in continuous culture under methanol-limiting conditions at a dilution rate of 0.2 h⁻¹. Subsequent enzyme assays and plasmid analysis showed that the *gdh* gene was stably maintained even in the absence of antibiotic selection and that the modified organism gave 4–7% higher carbon conversion than its unmodified precursor.

Discussion

The isolation of a fragment of *E. coli* DNA containing a functional gene for GDH and the identification of plasmid vectors capable of transferring this DNA fragment by conjugation from *E. coli* to the obligate methylotroph *M. methylotrophus* has enabled temperature-sensitive mutants of the latter organism to be screened for those deficient in GOGAT activity. The resultant transconjugants must use the *E. coli* GDH to synthesize all the glutamate² and hence all the organic nitrogen they require for growth at the non-permissive temperature. The use of the energetically more efficient GDH ammonia assimilation pathway results in more methanol being converted into cellular carbon. Thus, using recombinant DNA techniques, we have been able to make a fundamental alteration in the primary metabolism of *M. methylotrophus*, inactivating one function and replacing it with a heterologous one derived from a separate microbial genus. The resulting organism is able to convert significantly more methanol into SCP than the parental strain.

One of the principal problems of gene transfer between organisms which are not closely related has been in the expression of the transferred gene in its heterologous host¹². Although the *E. coli* *gdh* gene is expressed in *M. methylotrophus*, the enzyme activity produced per gene copy is 10–20-fold lower than in *E. coli*, as can be seen by comparing enzyme activities in

Table 3 Growth of *M. methylotrophus* and its derivatives on various ammonia concentrations

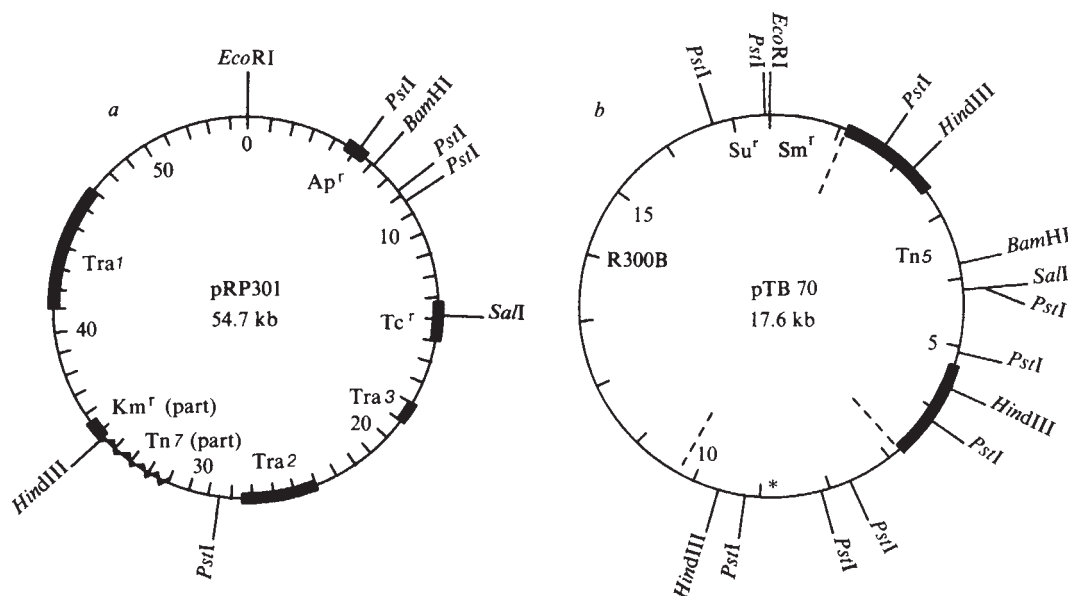
Incubation temperature (°C)	NH ₄ ⁺ concentration (mM)	Growth		
		<i>M. methylotrophus</i>	<i>M. methylotrophus</i> 56.2	<i>M. methylotrophus</i> 56.2 (pTB70/ <i>gdh</i>)
30	0.07	+	+	+
30	1	+	+	+
30	15	+	+	+
37	0.07	+	–	–
37	1	+	–	+
37	15	+	–	+

Growth was tested by streaking to single cells on 1.5% agar plates using the *M. methylotrophus* medium described in the legend to Table 1 except that the (NH₄)₂SO₄ concentration was varied as shown. Growth was scored after 48–72 h incubation. + Indicates visible colonies, – indicates no growth.

M. methylotrophus carrying pRP301/*gdh* or pTB70/*gdh* with those in a *gdh* mutant of *E. coli* carrying the corresponding plasmid (Table 1). In both microorganisms, the amount of GDH activity seems to be determined largely by gene copy number. In *E. coli* the relatively wide ranges of GDH activity which can occur suggests that any mechanism of regulating the level of *gdh* gene transcription can readily be overcome and that no significant deleterious effects accrue from excess *gdh* gene expression. In *M. methylotrophus* the presence of the *gdh* gene on the high copy-number plasmid pTB70 results in an enzyme activity which is sufficient to permit efficient growth under conditions where it provides the sole pathway of ammonia assimilation.

One surprising result of this work is the apparently high frequency with which we were able to detect mutants defective in GOGAT amongst mutants isolated solely on the basis of temperature sensitivity (4 out of 550). However, in both *E. coli* and *Klebsiella aerogenes* GOGAT has been shown to be a complex enzyme consisting of two different large polypeptides². Thus one might expect temperature-sensitive mutations

Fig. 4 Maps of the plasmid vectors used in *M. methylotrophus*. *a*, pRP301 was derived, as previously described⁹, from an RP4::Tn7 plasmid (pRP3) by excision of the small *Hind*III fragments, followed by ligation and transformation into *E. coli*. It has lost the Km^r of RP4 and the Tp^rSm^r of Tn7. Its remaining *Sal*I site is within the gene conferring Tc^r. The blocks represent previously mapped genes on RP4 (refs 9, 18). *b*, pTB70 was derived from R300B which is a wide host-range, multicopy, 8.5-kilobase pair, Sm^r Su^r IncQ plasmid^{19,20}. JR66a (IncIa, Tn5) was used to mobilize R300B from *E. coli* into *Proteus mirabilis* 13, selecting for Km^r on MacConkey agar containing polymyxin B (25 µg ml⁻¹) to counter-select the donor. IncI plasmids cannot be maintained in *P. mirabilis*²¹, therefore transconjugants should contain R300B::Tn5 plasmids. The restriction map of pTB70, a plasmid from one such transconjugant, is closely similar to that of R1162::Tn5, derived by Meyer *et al.*¹⁰ (R1162 is very similar or identical to R300B¹⁹), but shows that it has acquired, in addition to Tn5, a 3.6-kilobase pair piece of DNA (marked *) which may also be derived from JR66a (ref. 22). The heavy lines indicate the inverted repeats in Tn5. Restriction maps were deduced from the plasmid restriction fragments seen on 0.8% and 1.5% agarose gel after electrophoresis, using *Hind*III fragments of λDNA as size markers. Sizes given in kilobases (kb).



affecting this enzyme to occur at a relatively high frequency. Furthermore, the genome size of an obligate methylotroph such as *M. methylotrophus* may be relatively small (*Methanobacterium thermoautotrophicum*, an obligate methanogen, only has a genome of molecular weight 1.1×10^9 , as compared to the *E. coli* genome, for example, which has a molecular weight of 2.7×10^9 (ref. 13)).

For the purposes of strain improvement and modification, the alteration we have made in *M. methylotrophus* could not have been achieved either by prolonged selection under continuous culture conditions or by a conventional mutagenic approach. It represents the planned adaptation of a microorganism to a man-made environment using a combination of both mutation and intergeneric gene transfer. Effectively we have reversed the consequences of an evolutionary selection pressure which apparently demanded that the organism possessed a highly effective method of scavenging for ammonia in an ammonia-deficient environment. The evolution of many living systems

must have been subject to similar pressures from environments deficient in key nutrients. Where the energy source is non-limiting, such deficiencies may be accommodated by using more energy to push equilibria in favour of the synthesis of the desired product. For industrial or agricultural use, however, this form of adaptation is contrary to efficient production and we feel there are potential savings to be made by identification of metabolic features which incur such an 'energy penalty'. It represents a new and exciting area for the application of recombinant DNA techniques.

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The structure of one of the eight or more distinct chromosomal genes for human interferon- α

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The 12 interferon (IFN)-related sequences detected in a human gene bank fall into not less than eight distinct classes, indicating that there are at least eight IFN-related genes. Most, if not all, of these direct the synthesis of an IFN in Escherichia coli. The sequence of one chromosomal gene and its flanking regions was identical to that deduced for the cDNA corresponding to IFN- α 1 mRNA. No evidence was found for the existence of an intron; in either the coding or the non-coding segments of the gene.

WE recently reported the isolation of cloned hybrid plasmids containing cDNA derived from IFN-producing human leukocytes¹. Many of these plasmids were capable of directing the synthesis of polypeptides with human leukocyte-type IFN (HuIFN- α [ref. 2]) activity in *E. coli*^{1,3}. The cDNA inserts of two such hybrids, Z-pBR322(*Pst*)/HcIFN-2h and -SN206 (Hif-2h and Hif-SN206), have been sequenced^{3,4}. The amino acid sequences of the polypeptides IFN- α 1 and IFN- α 2, encoded by Hif-2h and Hif-SN206, respectively, differ in 17% of their positions; moreover, as deduced from the DNA sequences, IFN- α 1 is one amino acid longer than IFN- α 2, due to the absence of the codon for Asp 44 in IFN- α 2. IFN- α 1 and IFN- α 2 differ in five and nine of 32 amino-terminal residues, respectively, from IFN isolated from lymphoblastoid cells and partially sequenced by Zoon *et al.*⁵, designated IFN- α 3.

We concluded that at least three distinct genes for IFN- α (ref. 3) exist in man. It was of interest to find evidence for the presence of several IFN-related genes in the DNA of one individual, as the cDNA clones had been derived from pooled

blood samples and might reflect an extreme form of polymorphism. We therefore isolated and characterized cloned human chromosomal DNA fragments that hybridize or cross-hybridize to Hif-2h cDNA.

We report in this article the identification of eight distinct chromosomal DNA fragments which cross-hybridize with Hif-2h cDNA, most of which direct the formation of IFN in *E. coli*. We have identified and sequenced one chromosomal IFN gene which has the same coding sequence as Hif-2h cDNA. This gene for IFN- α 1 lacks introns, both in the regions corresponding to the coding and to the non-coding part of the mRNA. Other than the histone genes and certain viral genes, this may be the first instance of a functional gene in higher organisms which codes for a protein and yet contains no introns.

Cloned human chromosomal DNA fragments hybridizing to Hif-2h cDNA

Lawn *et al.*⁶ have prepared a collection of hybrid phage derived from fragments of fetal human chromosomal DNA, generated

by partial cleavage with *Hae*III and *Alu*I, and joined with *Eco*RI linkers to λ Charon 4A arms. This gene bank was screened by an 'in situ' procedure^{7,8} using as probe the ³²P-labelled IFN- α 1 cDNA insert excised from the hybrid plasmid Hif-2h^{1,4}. Sixteen hybridization-positive phage clones were isolated from 240,000 plaques by repeated plaque purification⁸. Ten of the hybrid phage DNA preparations were cleaved with *Hind*III, *Tac*I, *Hha*I, *Bam*HI, *Eco*RI and *Bgl*II, respectively, the fragments separated by agarose gel electrophoresis, transferred to a Millipore membrane⁹ and hybridized with the ³²P-labelled Hif-2h cDNA insert. Figure 1 shows, as an example, the patterns resulting from the experiments with *Eco*RI, *Pnd*III and *Bgl*II; Figure 2 summarizes the total data. In each case, the positions of at least a few characteristic restriction sites were established, and the region(s) hybridizing to the IFN probe were delineated.

The hybrid ML- λ Ch(*Eco*)/Hchr(AH)IFN-10 (chr-10) appears to contain two separate IFN- α 1-related genes, because both the 5'- and 3'-terminal Hif-2h cDNA probes (*Pst*-*Bgl*II fragment, nucleotides 1-315 for 5' probe; *Eco*-*Pst* fragment, nucleotides 685-910 for 3' probe, see ref. 4) hybridize with the 5.2- and the 20-kilobase *Bgl*-*Bgl* fragments (data not shown). Chr-16 clearly contains two IFN- α 1-related sequences, because the two *Bgl*II fragments comprising the λ arms and part of the insert hybridize to the probe, but two internal *Bgl*II fragments from within the insert do not. Clones chr-1 and chr-23 seem to be independent isolates of the same DNA segment, as they yield identical restriction fragments. Clones chr-3 and chr-26 may represent DNA segments which overlap over much of their

length because they have several *Eco*RI and *Hind*III fragments in common. The gene in chr-1 and one of the genes in chr-10 might be the same because the *Hind*III-*Hind*III and *Eco*-*Eco* fragments which hybridize with the Hif-2h cDNA probe have the same length (3.2 and 0.95 kilobases, respectively). It seems that the 'right-hand' gene of chr-16 may be identical with the gene in chr-35 (orientated in the opposite direction), as each of the two clones yields a 1.4-kilobase *Bgl*-*Bgl* and a 2-kilobase *Eco*-*Eco* fragment which hybridize with the Hif-2h cDNA probe. Therefore, the chr-16 and chr-35 inserts probably overlap. As Fig. 2 shows, all other hybridizing segments seem unique. For example, the characteristic features of the putative gene in chr-1 are: (1) *Eco*RI cleavage yields a strongly hybridizing 0.95-kilobase and a weakly hybridizing 7.8-kilobase fragment. Even if one of the hybridizing fragments has an *Eco*RI end generated by the cloning procedure, one of the two fragments must represent the chromosomal gene. (2) *Hind*III cleavage yields a weakly hybridizing >20-kilobase fragment, which must comprise the right-hand λ arm, and a strongly hybridizing 3.2-kilobase fragment, which must lie completely within the cloned fragment, and therefore represents the hybridizing region. (3) There is a *Tac*I site outside the hybridizing region, not further from the gene than about 5 kilobases. As mentioned above, chr-23 is identical to chr-1 in all regards, and one of the hybridizing regions of chr-10 contains a 0.95-kilobase *Eco*RI and a 3.2-kilobase *Hind*III fragment as does chr-1. The hybridizing *Hind*III fragments of chr-3, chr-12, chr-16, chr-30 and chr-35 are all completely contained in the cloned DNA frag-

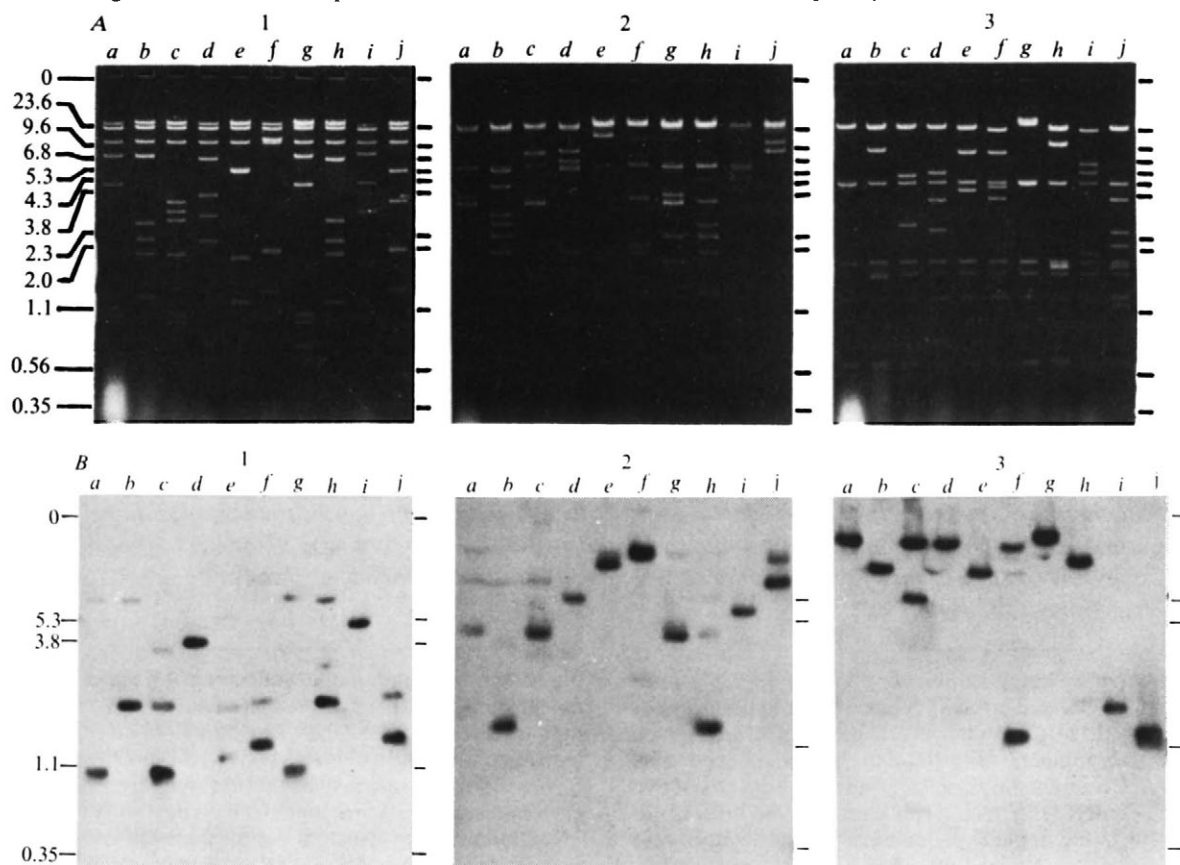


Fig. 1 Restriction and hybridization analysis of cloned human chromosomal IFN- α -related DNA. The IFN- α -related clones were selected from the λ -linked human genome library prepared by Lawn *et al.*⁶. About 240,000 plaques (about 3,000 plaques per 9.5 $\times$ 9.5 cm² filter membrane) were processed and screened by hybridization by the technique of Benton and Davis⁷ as modified by Maniatis *et al.*⁸. The probe was prepared by cleaving plasmid Hif-2h^{1,4} with *Pst*I, isolating the IFN cDNA insert by agarose gel electrophoresis, and labelling it by nick translation ([α -³²P]dATP, 250 Ci mmol⁻¹, and [α -³²P]dCTP, 200 Ci mmol⁻¹ (NEN); specific activity of probe, 1.5×10^8 c.p.m. μ g⁻¹) as described⁵⁰. Plaques appearing to give a positive hybridization response were subjected to three cycles of plaque purification⁸. Sixteen hybridization-positive clones were isolated and 10 of these were further examined. DNA was prepared^{51,52} and digested with the restriction enzymes indicated. All enzymes except *Bsp*I were purchased from New England Biolabs and used as recommended by the supplier, except that 200 μ g ml⁻¹ gelatin replaced bovine serum albumin in the buffers. The fragments were separated by electrophoresis in 1% agarose gels, using Tris-acetate-EDTA buffer (pH 7.8) containing 1.0 μ g ml⁻¹ ethidium bromide, and photographed in UV light. The fragments were transferred to Millipore membrane filter as described by Southern⁹ and hybridized with the nick-translated Hif-2h cDNA fragment described above. Panels 1, 2 and 3, digestion with *Eco*RI, *Bgl*II and *Hind*III, respectively. Hybridization (600,000 c.p.m. per filter) and washing procedures are as given by Maniatis *et al.*⁸. Exposure was for 12 h. **A**, Photographs of ethidium bromide-stained gels in UV light. **B**, Autoradiogram of the hybridized filters. The lanes contain the following samples: a, chr-1; b, chr-3; c, chr-10; d, chr-12; e, chr-13; f, chr-16; g, chr-23; h, chr-26; i, chr-30; j, chr-35. As a marker, *Hind*III-cleaved λ DNA and Hif-2h (ref. 4) cleaved with *Eco*RI and *Bgl*II were run in parallel.

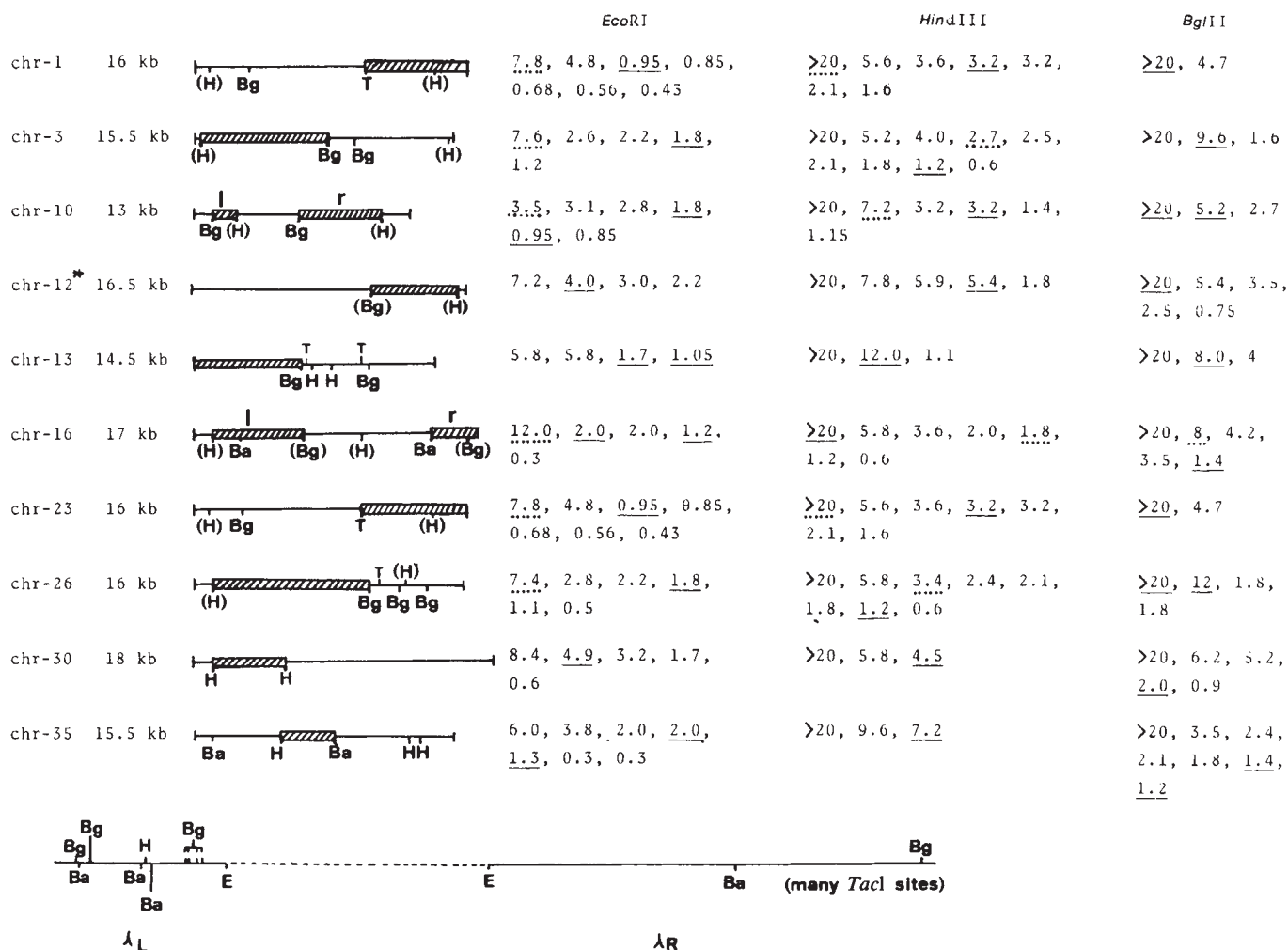


Fig. 2 Partial restriction maps of cloned, IFN- α -related chromosomal DNA fragments. Features of the vector λ Charon 4A DNA are shown in the bottom line. Derivation of the partial map of the chr-1 insert (a) was as follows: the total length of the insert, about 16 kilobases, was determined by summing the lengths of the *EcoRI* fragments. Cleavage with *BglII* yielded two fragments not found in the vector arms, with the lengths >20 and 4.7 kilobases. The larger fragment gave a positive hybridization response (compare with Fig. 1). As *BglII* cleavage of the λ arms must yield vector fragments of 1.55 (left arm) and 19.55 (right arm) kilobases linked to fragments of the insert, there must be a single *BglII* site within the insert, at 3.15 kilobases from the internal (*EcoRI*) end of the left λ arm. *HindIII* cleavage gave seven new fragments of >20, 5.6, 3.6, 3.2, 3.2, 2.1 and 1.6 kilobases, indicating six sites within the insert. The 3.2-kilobase fragment(s) gave a strong, and the >20-kilobase fragment a weak, hybridization response. As the (single) *HindIII* site on the left λ arm is 4.9 kilobases from the *EcoRI* site and the right arm (20 kilobases) has no *HindIII* site, one may conclude that the insert has a *HindIII* site at 0.7 kilobases from the internal end of the left arm. As the sum of all *HindIII* fragments derived from the insert alone is 13.7, and 0.7 kilobases of the 5.6-kilobase fragment are due to the insert, 14.4 kilobases of the insert are accounted for. Thus, about 1.6 kilobases of the >20-kilobase *HindIII* fragment must be due to the insert, which locates a *HindIII* site at about 1.6 kilobases from the internal end of the right-hand λ arm. The other four *HindIII* sites were not located. *TacI* digestion of the λ hybrid (data not shown) yielded 9.2- and 5.4-kilobase fragments, the smaller of which hybridized to the IFN- α probe; the λ arms were digested to fragments of ≤ 0.6 kilobases, except for one 1.1-kilobase fragment which is not derived from an internal end. Therefore, *TacI* digestion releases the insert with <0.6 kilobases of vector DNA at each end. As double digestion with *TacI* and *BglII* (data not shown) yielded two 5.4-kilobase and one 3.5-kilobase fragments, the *TacI* site must be located 5.4 kilobases to the right of the *BglII* site. Furthermore, the region hybridizing to the IFN probe must be located between the *TacI* site and the junction with the right-hand λ arm. The partial maps of the other cloned DNAs were derived in similar fashion; data additional to that of Fig. 1 were obtained from digests with *TacI* or *BamHI*, and double digests with *TacI* and *BamHI* and *HindIII*, respectively. The accuracy of the maps is about $\pm 10\%$. Parentheses around a restriction site indicate that other sites of the same kind exist but were not mapped. Hatched boxes indicate the locations of the DNA segment hybridizing to the probe. The table at the right indicates fragment length (other than those given by the λ arms alone) resulting from the cleavage with the restriction enzyme indicated. The fragments giving hybridization with the IFN- α probe are underlined with a solid line in the case of a strong, and with a dotted line in the case of a weak, response. Bg, *BglII* site; H, *HindIII* site; Ba, *BamHI* site; E, *EcoRI* site; T, *TacI* site. kb, kilobases. * Chr-12 has two unmapped *TacI* sites.

ment and all differ from the 3.2-kilobase fragment of chr-1. The hybridizing *HindIII* fragment of chr-13 is 12 kilobases long; 7.1 kilobases lie within the insert, so that here too the 3.2-kilobase fragment of chr-1 cannot be accounted for. With chr-16, two hybridizing regions were delineated (see above). The right-hand putative gene (r), which is probably not completely contained in the fragment, is considered identical to that in chr-35. As the hybridizing region of chr-35 has no features in common with chr-1, chr-16 (r) is also different from chr-1. The hybridizing *HindIII* fragment of chr-16 (l) is completely contained in the insert and is far smaller than that of chr-1. We conclude that the putative genes of chr-3, chr-12, chr-13, chr-16, chr-26, chr-30

and chr-35 differ from that of chr-1. The same conclusion is reached from the fact that none of the *EcoRI* fragments of chr-1, hybridizing or not, are present in any of the clones mentioned above except for a 7.6-kilobase fragment of chr-3 which is close in size to the 7.8 kilobases of chr-1. From similar arguments based on the data of Fig. 2 regarding comparisons of the other clones and additional Smith-Birnsteil mapping of chr-3, chr-10, chr-13, chr-26 and chr-35, we conclude that the 12 putative IFN- α genes fall into not less than eight distinct classes.

Hif-chr-35 (and the right-hand segment of Hif-chr-16, to which it is probably identical) is the only DNA with a *BglII* site in the putative coding region (compare with table in Fig. 2). As

the two different IFN- α cDNAs examined to date, Hif-2h and Hif-206, have one and two *Bgl*II sites, respectively, within their coding sequences, it seems likely that only the chr-35 gene with its single *Bgl*II site is a counterpart to one of the two cloned cDNAs, most likely to Hif-2h. This conclusion was reinforced by observing that when the 3'-terminal Hif-2h cDNA segment, which contains only the 3' non-coding region, was used as a probe, the chr-35 restriction fragments gave a much stronger hybridization response than those of any of the other chromosomal DNAs. As 3' non-coding regions of homologous genes diverge more strongly than their coding sequences^{3,10}, a 3' probe discriminates between different but related sequences

more readily than total probes. The chromosomal counterpart to Hif-206 has not yet been found.

How many of the chromosomal clones contain sequences coding for polypeptides with IFN activity? As the cytopathic effect reduction assay¹¹ can detect minute amounts of IFN, less than one active molecule per bacterial cell, we tested the lysates of *E. coli* infected with the hybrid λ phages. Eight of 10 phage strains examined (all but chr-10 and chr-12) gave lysates containing IFN activity, ranging from 3 to 50 U ml⁻¹. For chr-10 and chr-12, the hybridizing *Hind*III-*Hind*III or *Eco*-*Eco* fragments, subcloned into the *Pst* site of pBR322, expressed IFN activity in *E. coli* (K. Henco and M. Streuli, unpublished results).

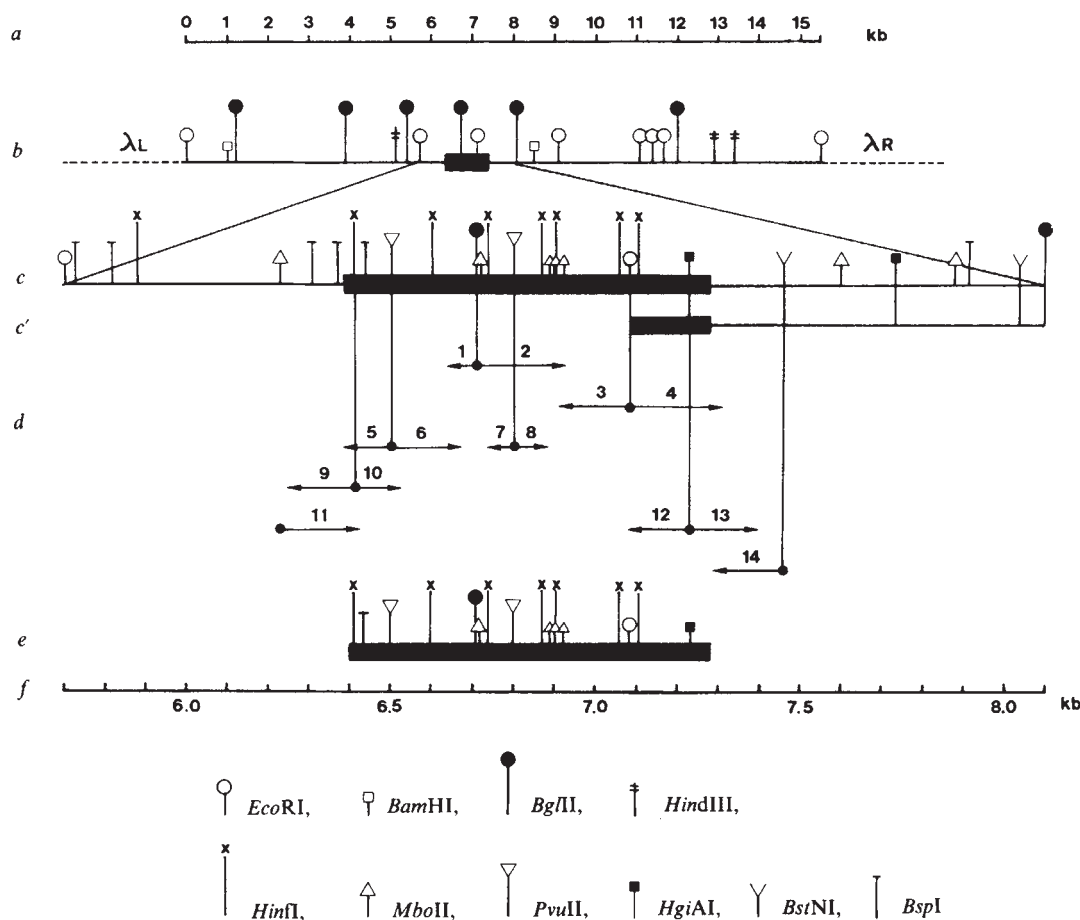


Fig. 3 Restriction map of the cloned human chromosomal DNA fragment chr-35; strategy for the nucleotide sequence determination. The gene-containing region of chr-35 was subcloned: chr-35 was cleaved with *Hind*III and *Bam*HI, and the 3.4-kilobase fragment (which contains the region hybridizing to the Hif-2h cDNA probe, compare with Fig. 1) was elongated with dC residues, annealed to pBR322 which had been cleaved with *Pst*I and elongated with dG¹³. Clones of *E. coli* HB101 transformed with the resulting hybrids were screened by *in situ* colony hybridization⁵³ with the ³²P-labelled Hif-2h fragment described in Fig. 1 legend. Plasmids (Z-pBR322(*Pst*)/HchrIF-35HB or HchrIF-35HB) were prepared from the positive clones by method B described by Wilkie *et al.*⁵⁴. The orientation of the insert was established by digesting the plasmid with *Eco*RI and determining the length of the shorter fragment. The orientation coinciding with that of the β -lactamase gene was designated α , the opposite one, β . Restriction sites were determined by Smith-Birnstein mapping⁵⁵: HchrIF-35HB α was digested with *Eco*RI, labelled at the 5' termini and digested with *Bgl*II (and *Pst*I, to cleave an undesired fragment of about 1 kilobase). The 1.04-kilobase *Eco*-*Bgl*II (3'-proximal) and the 0.96-kilobase (5'-proximal) *Eco*-*Bgl*II fragments were isolated by agarose gel electrophoresis as described⁵⁶. Both fragments were partially cleaved with *Hinf*I, *Bsp*I and *Mbo*II, respectively, the products separated on a 1% agarose gel in Tris-acetate buffer (pH 7.8) containing 1 μ g ml⁻¹ ethidium bromide, and radioactive bands were visualized by autoradiography (data not shown). *Bst*NI and *Hgi*AI sites were similarly determined only on the 1.04-kilobase (3'-proximal) *Eco*-*Bgl*II fragment (c'). a, Scale in kilobase pairs (kb) for b; b, restriction map of chr-35 DNA in Charon 4A DNA; c, blow-up of restriction site map of the IFN coding sequence and flanking regions determined on HchrIF-35HB α ; e, map of the cloned cDNA insert of Hif-2h (from Mantei *et al.*⁴); f, scale in kilobase pairs applicable to c-e. For nucleotide sequence determination, HchrIF-35HB α was cleaved with the restriction enzyme indicated and 5'-terminally labelled as described by Mantei *et al.*⁴. Labelled fragments were cleaved with a second restriction enzyme, the products were separated by electrophoresis through a 5% polyacrylamide gel in Tris-borate-EDTA buffer⁵⁷, extracted from the gel and purified as described⁵⁶. The various fragments for sequencing were prepared as follows (the number indicates the nominal fragment chain length in base pairs, the ends are designated by the enzyme which generates them, the labelled end is indicated by an asterisk, and the numbers in parentheses refer to the arrows shown in d). (1) and (2), Cleavage of HchrIF-35HB α with *Bgl*II, labelling, cleavage with *Eco*RI and *Pst*I, isolation of (1) *Bgl**-*Eco*-940, and (2) *Bgl**-*Eco*-360. (3) and (4), Cleavage of HchrIF-35HB α with *Eco*RI, labelling, cleavage with *Bsp*I, isolation of (3) *Eco**-*Bsp*-680 and (4) *Eco**-*Bsp*-880. (5), (6), (7) and (8), Cleavage of HchrIF-35HB α with *Pvu*II, labelling, cleavage with *Bgl*II and *Eco*RI, isolation of (5) *Pvu**-*Eco*-780, (6) *Pvu**-*Bgl*-215, (7) *Pvu**-*Bgl*-90 and (8) *Pvu**-*Eco*-290. (9) and (10), Cleavage of HchrIF-35HB α with *Eco*RI, isolation by 1% agarose gel electrophoresis in Tris-borate EDTA buffer⁵⁷ of *Eco*-*Eco*-1300, cleavage with *Hinf*I, isolation of *Hinf*-*Hinf*-450 (for [9]) and *Hinf*-*Hinf*-180 (for [10]), labelling, cleavage with *Mbo*II (for [9]) or *Ava*II (for [10]), isolation of (9) *Hinf**-*Mbo*II-190 and (10) *Hinf**-*Ava*II-150. (11), Cleavage of HchrIF-35HB α with *Mbo*II, labelling, cleavage of HchrIF-35HB α with *Mbo*II, labelling, cleavage with *Bgl*II, isolation of (11) *Mbo**-*Bgl*-465. (12), (13) and (14), Cleavage of HchrIF-35HB α with *Bsp*I and *Bgl*II, isolation by agarose gel electrophoresis as above of *Bsp*-*Bgl*-1200, cleavage with *Hgi*AI (for [12] and [13]), or *Bst*NI (for [14]), labelling, cleavage with *Mbo*II (for [12] and [13]) or *Eco*RI (for [14]), isolation of (12) *Hgi**-*Mbo*II-300, (13) *Hgi**-*Mbo*II-360, and (14) *Bst**-*Eco*-380.

This shows that all cloned DNA segments contained at least one potentially active IFN gene, and that these genes most likely did not contain introns in their coding regions, as *E. coli* is believed to be incapable of splicing mRNAs¹². Our data thus suggest that the genome of an individual human contains not less than eight different IFN-coding sequences, supported by the fact that the proportion of IFN-related sequences detected in the clone bank is about one in 16,000. Assuming a value of 3×10^9 base pairs for the haploid human genome, the expected value for a single-copy gene in a bank with an average DNA fragment size of 16 kilobases (the average value found for the 10 clones examined) would be about one in 190,000; thus, the frequency of Hif-2h-related segments is 12 times higher than expected for a single gene. When Lawn *et al.*⁶ screened 300,000 plaques from the same gene bank with a β -globin cDNA probe, they identified two clones. The expected value for a single gene should have been 1.6 clones per 300,000 plaques, however the probe could potentially cross-hybridize with two γ and a δ gene as well. We conclude that the number of distinct DNA segments cross-hybridizing with IFN- α 1 cDNA may be around 10–15, and that many of these will prove to code for IFN-like proteins.

Identification of a chromosomal gene corresponding to IFN- α 1

The cDNA insert of Hif-2h (which codes for IFN- α 1) has a single *Bgl*II site within the coding region⁴. The chromosomal IFN-related DNA clone with the same feature was chr-35. This DNA was further characterized by restriction analysis and Southern blotting; Fig. 2 shows that the region hybridizing to the Hif-2h probes lies within a 3.4-kilobase region flanked by a *Bam*HI and a *Hind*III site. To facilitate further analysis, this fragment was subcloned into the *Pst*I site of pBR322 using the dC-dG tailing procedure¹³. Several clones of *E. coli* transformed with the resulting hybrids were isolated, cultures were grown to an apparent OD₆₅₀ of 0.8 and the bacteria collected and lysed as described¹. Seven of 10 clones examined showed IFN activities of 75–500 U per g of cells. One of the IFN-producing clones, Z-pBR322(*Pst*)/Hifchr35-HB α , was characterized by restriction analysis. Figure 3 shows that its restriction map is indistinguishable from that of the cDNA insert of Hif-2h; in particular, there is no indication of introns within the coding sequence, that is, between the *Hinf*I site in the 5' non-coding region, and the *Eco*RI site in the 3' non-coding region.

To prove this conclusion, and to determine whether or not the 5' and 3' non-coding regions contain introns, it was necessary to compare the nucleotide sequences of Hif-2h cDNA and the relevant segments of the chr-35 DNA.

Nucleotide sequences of the chromosomal gene chr-35 and the cDNA coding for IFN- α 1

The nucleotide sequence of Hif-2h cDNA has been reported previously⁴. The coding region, which includes a signal sequence, comprises 567 base pairs and is followed by a 242-base pair 3' non-coding region. The 5' non-coding region consists of 56 nucleotides and is probably incomplete, as with all cDNA clones generated by the technique we used¹⁴. Moreover, it has been found that artifactual sequences can be generated in the process of synthesizing the double-stranded cDNA copy of a mRNA, especially in the 5' region¹⁵. We therefore redetermined and extended the 5'-proximal nucleotide sequence essentially as described by Tsujimoto and Suzuki¹⁶. We prepared a *Pvu*II-*Bsp* minus-strand restriction fragment, extending from nucleotide +44 to nucleotide +5 (see Fig. 3) and labelled at the *Pvu*II end. This fragment was hybridized to poly(A) RNA from IFN-producing leukocytes and reverse transcriptase was used to elongate it with unlabelled deoxyribonucleotides. The denatured product, analysed on a polyacrylamide gel (Fig. 4), showed a major band of about 115 nucleotides. Thus, about 75 nucleo-

tides had been added to the primer, indicating a 5' non-coding sequence of about 70 nucleotides. The major radioactive band was sequenced by the Maxam-Gilbert procedure¹⁷ and the sequence shown in Fig. 6 obtained. The sequence of the cDNA transcribed directly on the mRNA is identical to that of the cloned cDNA, except for the four 5'-terminal nucleotides; moreover, it is longer by 11 residues. As at least two species of IFN- α are expressed in human leukocytes, the mRNA species which served as template for direct reverse transcription may differ from that which gave rise to the cloned cDNA. However, as shown below, the chromosomal DNA is identical to the cloned Hif-2h cDNA except for the four discrepant nucleotides,

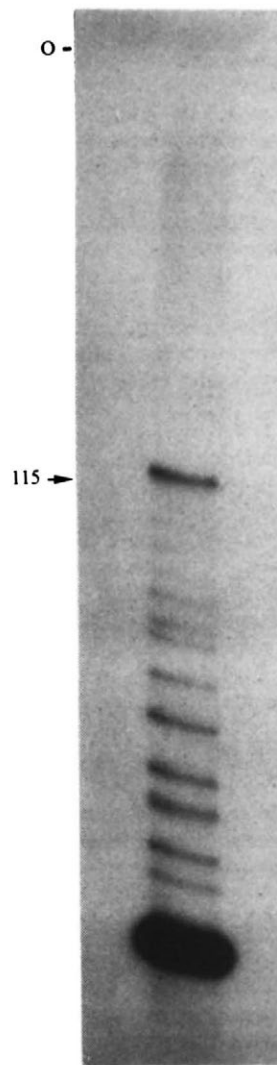


Fig. 4 Determination of the nucleotide sequence of cDNA complementary to the 5' end of IFN mRNA. Synthesis of cDNA complementary to the 5' end of IFN mRNA was accomplished as follows: the plasmid Hif-2h, which contains the almost complete cDNA to IFN- α 1 mRNA, was cleaved with *Pvu*II and the 5' termini labelled with ³²P-phosphate as described⁴, to a specific activity of 1.2 μ Ci pmol⁻¹. Following cleavage with *Bsp*I, the products were denatured and applied to a 16% polyacrylamide gel for strand separation¹⁷. The 40-base pair *Pvu*II-*Bsp* minus-strand fragment (which extends from positions 44 to 5, see Fig. 6) was recovered as described¹⁶. The fragment (0.5 pmol, 0.5 μ Ci) was mixed with 3 μ g 12S poly(A) RNA from virus-induced human leukocytes¹ in 4.5 μ l NaCl (6.6 mM), 0.6 mM EDTA, 6.6 mM HEPES (pH 7.5). After heating for 90 s to 100 °C, NaCl was added to 0.18 M and the mixture (5 μ l) incubated 40 min at 60 °C. After addition of Tris-HCl (pH 7.5) to 40 mM, dithiothreitol to 1 mM, MgCl₂ to 5 mM, dGTP, dATP, TTP, and dCTP to 0.5 mM each (final volume 30 μ l), incubation was carried out with 3.5 U reverse transcriptase for 90 min at 37 °C. The mixture was extracted with phenol, precipitated with ethanol, and the DNA subjected to electrophoresis through a 12% polyacrylamide gel in 50 mM Tris-borate (pH 8.3), 1 mM EDTA, 7 M urea, for 5 h at 900 V. Autoradiography was for 21 h. The band with the lowest mobility (corresponding to a fragment about 115 nucleotides long) was eluted and sequenced as described^{4,17}. The sequence is given in Fig. 6.

where it agrees with the direct reverse transcript. Thus, the discrepancy is likely due to the type of artefact described by Richards *et al.*¹⁵.

Does the direct reverse transcript extend to the 5' terminus of the mRNA as Tsujimoto and Suzuki¹⁶ found? The case of IFN- α 1 was examined using an S₁ mapping procedure. A 780-base pair *PvuII*-*EcoRI* restriction fragment of the chromosomal DNA, which extends from position +44 to -740 and comprises the region corresponding to the putative 5' terminus of the mRNA, was prepared with a 5'-terminal label at the *PvuII* end, hybridized to poly(A) RNA from IFN-producing leukocytes and digested with S₁ nuclease. As a size marker, a sample of the labelled probe was degraded by the Maxam-Gilbert procedure¹⁷. Polyacrylamide gel analysis revealed two major protected fragments (Fig. 5) of 115 and 116 base pairs, and a series of minor, shorter fragments. Mouse β -globin mRNA, analysed by this procedure, yields two DNA fragments longer by four and five nucleotides than expected¹⁸, perhaps because the presence of a cap hinders digestion of the DNA probe by S₁ nuclease. We conclude that the position of the 5'-terminal nucleotide of the major portion of the mRNA lies about 67 nucleotides upstream from the first AUG triplet, agreeing with conclusions drawn from the analysis of the direct cDNA transcript. We tentatively assume that the 5' end of the IFN- α 1 mRNA has the sequence AG... and begins at position -67 (Fig. 6); however, it could equally map at the AG two nucleotides further upstream. Most eukaryotic mRNAs start with the sequence AC... (ref. 19), however the rat preproinsulin II mRNA is believed to begin with the sequence AG²⁰. A faint set of bands, mapping 98 base pairs upstream (about 5% of the radioactivity of the major bands) was also detected. These are probably due to incompletely digested probe, as similar findings have been noted occasionally with the globin mRNA system; however we cannot yet exclude a longer species of IFN mRNA, perhaps a precursor.

Some restriction sites of the chromosomal IFN subclone Hif-chr35Hb α were mapped, and the nucleotide sequence corresponding to the gene and its flanking regions determined as outlined in Fig. 3 legend. Comparison of the chromosomal and the cDNA sequence (Fig. 6) shows complete identity, from the 5' end of the cDNA sequence obtained by direct reverse transcription to the last nucleotide preceding the poly(A) tail, as determined from the cloned cDNA. Thus, no intron could be detected in the chromosomal sequence corresponding to mature IFN mRNA.

Is the IFN- α 1 gene devoid of introns?

The IFN- α 1 primary transcript could initiate upstream from the position corresponding to the 5' end of the mature mRNA, and an intron could be contained within this segment of the precursor. Maturation of the pre-mRNA would then require splicing, followed by removal of a 5' segment. It will be interesting to determine whether the weak bands detected by S₁ mapping are due to IFN- α 1 precursor transcripts longer than mature IFN- α 1, and whether or not mature IFN mRNA is capped: *in vitro* transcription of the chromosomal gene might provide further clarification. Anyway, note that 28 nucleotides upstream from the putative 'cap' site there is a sequence 5'-TATTTAA-3' flanked on both sides by GC-rich segments, which may correspond to the 'Hogness box' described 23 to 25 nucleotides upstream from the cap site for many chromosomal genes²¹. This suggests that initiation of transcription does occur at the putative cap site, and that there are no introns within the IFN- α 1 transcriptional unit. Certainly there are no introns within the IFN- α 1 coding sequence, and the fact that most, if not all, chromosomal IFN genes identified so far lead to the synthesis of IFN in *E. coli* suggest that this is generally true for IFN- α sequences. In this regard the IFN- α 1 (and possibly all IFN- α) genes seem to resemble the sea urchin histone genes which also have no introns²², and to differ from all other protein-coding nuclear genes of higher eukaryotes described so far, which have at least one, but frequently many, introns²³⁻²⁵.

Messenger RNAs of SV40 accumulate in the cytoplasm only if the corresponding primary transcripts contain an intron^{26,27}. Moreover, Hamer and Leder²⁸ have shown that β -globin coding sequences cloned in SV40 DNA only give rise to stable mRNAs if the primary transcript contains an intron, either in the SV40 or in the globin moiety. This cannot be true for all coding transcripts, as neither do histone genes have introns²², nor does the primary transcript of the polypeptide IX gene of adenovirus type II contain an intron counterpart²⁹. Also, much of retroviral RNA is exported from the nucleus without splicing^{30,31}. Since 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) inhibits the synthesis of most mRNAs, but not that of the adenovirus polypeptide IX mRNA³², it will be of interest to determine its effect on IFN- α mRNA synthesis.

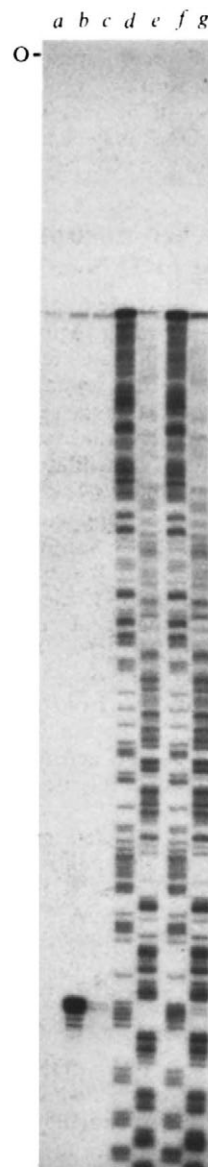


Fig. 5 Mapping of the 5' end(s) of IFN mRNA by S₁ mapping. The plasmid Z-pBR322(*Pst*)/HchrIF-HB α was cleaved with *PvuII* and the 5' termini labelled with ³²P-phosphate⁴. After *EcoRI* cleavage, the ³²P-labelled 780-base pair fragment was recovered by agarose gel electrophoresis. The fragment (0.03 pmol, 24,000 c.p.m.) was denatured, hybridized in 80% formamide to poly(A) RNA from IFN-producing leukocytes¹, digested with nuclease S₁ (ref. 58), and analysed by polyacrylamide gel electrophoresis on a 12% gel (18 h, 900 V), all as described in Weaver and Weissmann¹⁸ and Mantei *et al.*⁴. To provide a 'sequence ladder' as length marker, the ³²P-labelled fragment described above was subjected to the A+G and C+T degradations described by Maxam and Gilbert¹⁷ and in protocols provided by the same authors. The autoradiogram shown was obtained after a 5-day exposure. Lane a, 0.1 μ g poly(A) RNA from non-induced leukocytes; lane b, 2 μ g induced leukocyte RNA; lanes c, f and g, 0.2 μ g induced leukocyte RNA; lanes d and f, A+G degradation products; lanes e and g, C+T degradation products.

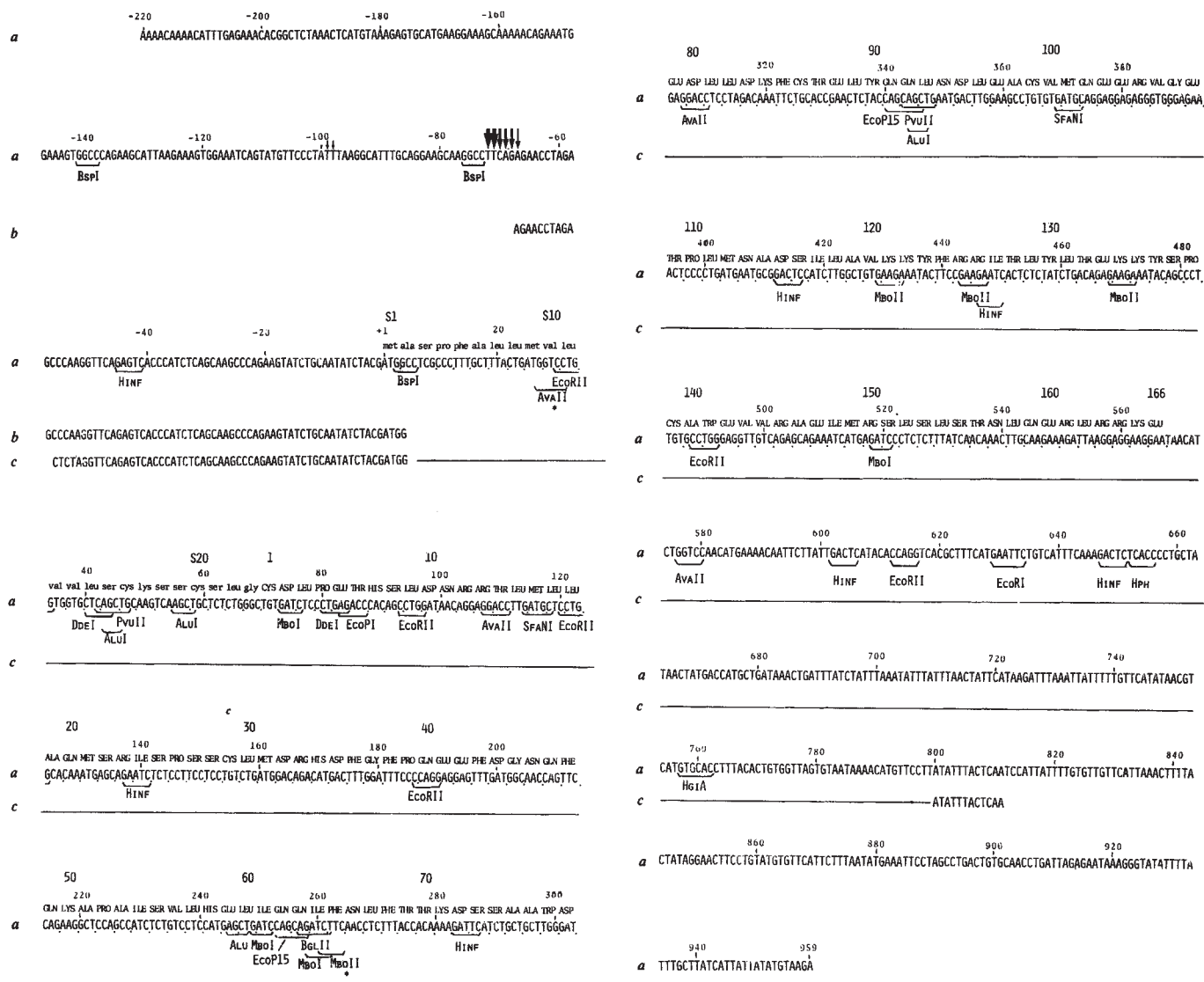


Fig. 6 Nucleotide sequence of the chromosomal IFN- α 1 coding segment and its flanking regions. *a*, The 5'-labelled fragments described in Fig. 3 legend were degraded according to Maxam and Gilbert¹⁷ with the modifications described in protocols provided by the same authors in September 1978 and fractionated as described in ref. 4. *b*, The sequence of cDNA complementary to IFN- α 1 mRNA determined as described in Fig. 4 legend. *c*, Nucleotide sequence of the cloned IFN- α 1 cDNA (Hif-2h, from Mantei *et al.*⁴); the continuous line indicates identity with the sequence in *a*. The vertical arrows indicate the location (± 1 nucleotide) of the probe termini generated by the S₁ RNA mapping procedure, as described in Fig. 5 legend.

The multiplicity of interferon genes

We have shown that chromosomal IFN genes isolated from a gene bank derived from an individual embryo fall into at least eight different classes, as judged by restriction site distribution. As there is more than one difference between the classes in almost all cases, the genes probably differ by more than the occasional point mutation, as is commonly found with polymorphic genes^{33,34}. As the cloning procedure may introduce deletions into chromosomal DNA³⁵, a conclusive classification requires confirmation by analysis of independent gene isolates and/or Southern analysis of uncloned chromosomal DNA. We believe that all the genes we have described are of the α (or leukocyte) type because (1) the homologies between α and β (or fibroblast) type IFN are so limited that cross-hybridization is unlikely (ref. 36 and W. Fiers personal communication) and (2) because all IFN- β cDNAs analysed so far contain a *Bgl*II site³⁷⁻³⁹, and the only chromosomal IFN gene with a *Bgl*II site in the set analysed (chr-35) is definitely an α -type gene.

The genes we analysed could be derived from at least four different loci assuming that substantial polymorphism exists, but more likely reflect the existence of eight different loci. A linkage map will resolve this question; so far, there is evidence that at

least some of the genes are clustered, for example chr-10 and chr-16 each contain two IFN sequences.

Are all IFN genes expressed in eukaryotic cells? As most, if not all, chromosomal IFN genes identified so far give rise to biologically active IFN in *E. coli*, they obviously are potentially expressible and do not resemble the α -globin-like silent gene found by Nishioka *et al.*⁴⁰. Clearly, analysis of the IFN fractions derived from leukocyte IFN preparation by HPLC⁴¹ may reveal the number and nature of IFNs produced by these cells. The heterogeneity of leukocyte IFN preparations⁴¹ often ascribed to varying degrees of glycosylation may reflect the gene multiplicity described here. As an IFN species isolated from lymphoblastoid cells and partially sequenced⁵ (IFN- α 3) differs from two species of IFNs known to be expressed in virus-treated leukocytes (IFN- α 1 and IFN- α 2), conceivably different types of cells may preferentially express different IFN α genes. The easiest analyses are probably at the mRNA level, using as probes the 3' non-coding regions of the different IFN- α genes, which seem to differ the most between different genes³.

We conclude that at least eight but perhaps as many as 10–15 distinct IFN genes exist. This gene multiplicity suggests that

IFNs may be functionally heterogeneous. The most striking functionally heterogeneous proteins encoded by a multigene family are the immunoglobulin variable chains, where each gene product probably has a different epitope specificity. The human β -globin family comprises at least five members, some of which differ distinctly in their biochemical properties, and which are synthesized at different stages of development^{42,43}. With other families, such as those of the chorion proteins^{44,45} or actins⁴⁶, it is not clear to which extent the genes code for proteins of different functions. The differences in amino acid sequences of at least some of the IFN genes³⁻⁵ are certainly substantial enough to allow for proteins with quite different properties. As reported elsewhere, one striking functional difference between IFN- α 1 and IFN- α 2 is their relative virus-protective activity on cells of

different species: the ratio of activities on bovine and human cells is 20–50 for IFN- α 1 and one or less for IFN- α 2 (refs 3, 47). This difference in cross-species reactivity could reflect different specificity for cell receptors, and questions whether different IFN species may be addressed to different target cells within the same host. It will also be of interest to ascertain whether each of the IFN species shows the full range of activities ascribed to leukocyte IFN preparations^{39,48}.

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A family of structural genes for human lymphoblastoid (leukocyte-type) interferon

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Amino acid sequences of tryptic and chymotryptic peptides from human lymphoblastoid interferon (IFN- α) have been determined. The results show that IFN- α consists of a family of proteins with at least five different, but homologous, primary structures. There appears to be little, if any, glycosylation of the major components of IFN- α .

TWO major classes of human type I interferons (IFNs), leukocyte (IFN- α) and fibroblast (IFN- β), may be distinguished serologically and have been shown to be the products of separate structural genes¹. Amino-terminal peptide sequences showed no homology^{2,3}, although there is homology elsewhere in the sequences⁴. Interferon produced by the Namalwa lymphoblastoid cell line after induction by Sendai virus is primarily of the leukocyte type⁵. Human leukocyte (and Namalwa) IFN is heterogeneous, as shown by SDS-polyacrylamide gel electrophoresis^{6–10}, isoelectric focusing^{11–16}, high-performance liquid chromatography¹⁷ and affinity chromatography⁹. This heterogeneity has been ascribed to different extents of glycosylation of the molecule^{7,9,15,18,19} or partial degradation of a

native molecule^{8,20}. We show here that IFN- α produced by Namalwa cells consists of a family of proteins with at least five different, but homologous, amino acid sequences, and is therefore the product of several structural genes. No direct evidence for glycosylation has been found, although it cannot, at present, be excluded.

Separation of interferons

Studies on the structures of human IFNs have been limited by the small amounts of pure material available. However, Namalwa IFN, produced on a large scale at Wellcome Research Laboratories²¹ and purified by a combination of methods including trichloroacetic acid precipitation, acid ethanol extrac-

tion and antibody affinity chromatography, was available in relatively large quantities. About 4 mg was used in the present work. The IFN was separated into two forms by chromatography on a column (10×1,980 mm) of Sephadex G-75 (Superfine) in NH_4HCO_3 (50 mM)/0.01% thiodiglycol. Each form (IFN- α A and IFN- α B, of apparent molecular weights 22,000 and 18,500, respectively) gave multiple bands upon SDS-gel electrophoresis (Fig. 1).

Sequencing of interferons

Amino acid compositions of IFN- α A and IFN- α B were determined, and were found to be similar to each other, but significantly different from those reported for human leukocyte¹⁷ and one component of lymphoblastoid²² IFNs. Less than 0.2 residues of glucosamine or galactosamine per molecule were determined in either IFN- α A or IFN- α B. Details of these studies will be reported elsewhere (G. A., manuscript in preparation).

Tryptic peptide maps of performic acid-oxidized IFN- α A and IFN- α B were prepared on cellulose thin-layer plates by electrophoresis at pH 4.4 and chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10 by vol). Peptides were detected with ninhydrin and arginine-containing peptides with phenanthrene quinone²³. The maps were quite different: only free lysine and arginine and one other major spot in each clearly coincided, although some weak spots on the map of IFN- α B corresponded to some of the strong spots on the map of IFN- α A. The number of tryptic peptides from IFN- α A indicated that this fraction of IFN consisted of a single polypeptide of molecular weight about 18,000, with, perhaps, limited microheterogeneity; in contrast, more peptides were present in the tryptic digest of IFN- α B than would be derived from a single polypeptide chain of this size.

Amino-terminal sequences of IFN- α A and IFN- α B (about 3 nmol each) were determined by the method of Chang *et al.*²⁴, using 4-*N,N*-dimethylaminoazobenzene-4'-isothiocyanate. The results for the 14 N-terminal residues were similar:

IFN- α A (–)–Asp-Leu-Pro-Glx-Thr-His-Ser-Leu-Gly-Ser–(–)–Arg-Thr-

IFN- α B (–)–Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Ser^{Asn}–Arg-Arg-Thr-Ser

The first six steps of the degradation gave very clear results (except that some glutamic acid as well as glutamine seemed to be present at position 5 in IFN- α A), showing that any impurities with a free N-terminal residue were present at less than 5% of the molar amount of IFN. No N-terminal residue was detected for either IFN- α A or IFN- α B. The N-terminal residue was identified as cysteine from the study of peptides from the reduced and [S - ^{14}C]carboxymethylated protein. As little butyl acetate-soluble coloured material was released upon treatment of the dimethylaminoazobenzene thiocarbonyl proteins with trifluoroacetic acid, the N-terminal cysteine residue in the native protein is probably involved in a disulphide bond.

Tryptic peptides from IFN- α A and tryptic and chymotryptic peptides from IFN- α B were purified from ~1 mg digests of the performic acid-oxidized or reduced and ^{14}C -carboxymethylated proteins by gel filtration followed by thin-layer peptide mapping. Peptide sequences were determined by either the micro-'dansyl-Edman' method²⁵ or the method of Chang *et al.*²⁴.

Comparison of interferon sequences

While this work was in progress, a complete amino acid sequence, deduced from the nucleotide sequence of cloned cDNA derived from leukocyte IFN mRNA, was published²⁶. Peptide sequences determined here could be aligned unambiguously by homology with the sequence of Mantei *et al.*²⁶, and a summary of the directly determined peptide sequences is presented in Fig. 2. Many differences between the sequences of IFN- α A, IFN- α B and that presented in ref. 26 are evident. The N-terminal 20 residues determined by Zoon *et al.*² are represented among the sequences determined here, except that

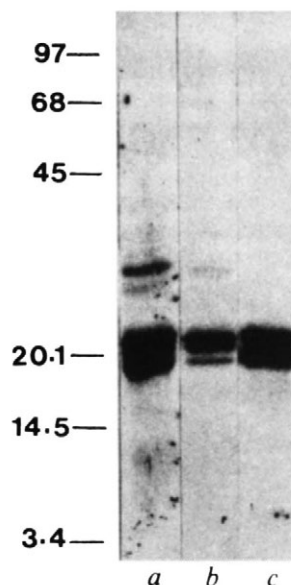


Fig. 1 SDS-gel electrophoresis of Namalwa IFN. Samples (~5 μg) of Namalwa IFN (a), IFN- α A (b) and IFN- α B (c) were submitted to SDS-polyacrylamide gel electrophoresis (15% acrylamide) using the system of Laemmli³², but with slab rather than tube gels. The gel was fixed in 15% trichloroacetic acid, stained with 0.1% Coomassie blue R250 in methanol/acetic acid/water (45:10:45 by vol) overnight and destained in 7% acetic acid. The positions of marker proteins run in parallel are shown: 97, phosphorylase; 68, bovine serum albumin; 45, ovalbumin; 20.1, soybean trypsin inhibitor (Kunitz); 14.5, lysozyme; 3.4, insulin B chain.

no N-terminal serine was detected. Residues 72–113 of IFN- α A were isolated as a single tryptic peptide, and only the first 19 residues of this could be sequenced. Sequence information has been obtained for all but five residues of IFN- α B. Several peptides were isolated in yields too low to permit sequencing, and other peptide sequences will probably be identified with further study.

As predicted from the tryptic peptide map, IFN- α A consists of an almost homogeneous peptide chain, but microheterogeneity was observed at a few positions. IFN- α B is a mixture of at least three different polypeptides (all different from both of those in IFN- α A), as shown most clearly by the isolation of three different tryptic peptides from residues 14–23 and from residues 24–31:

14 23
Thr-Leu-Met-Leu-Leu-Ala-Gln-Met-Ser-Arg,
14 22
Thr-Leu-Met-Leu-Leu-Ala-Gln-Met-Arg,
14 23
Ala-Leu-Ile-Leu-Leu-Ala-Gln-Met-Gly-Arg, and
24 31
Ile-Ser-Pro-Ser-Ser-Cys-Leu-Met-Asp-Arg,
24 31
Ile-Ser-Pro-Phe-Ser-Cys-Leu-Lys,
24 31
Ile-Ser-Leu-Phe(Ser, Cys)Leu-Lys.

High yields of C-terminal peptides were obtained from both IFN- α A and IFN- α B, showing that proteolysis close to the C terminus does not occur during the biosynthesis and isolation of lymphoblastoid IFN.

The total number of different polypeptide sequences cannot be determined from the present work, but the minimum number is five (at least two in IFN- α A and at least three in IFN- α B). The complete separation of the polypeptide components which would be required for the determination of the precise number of different total sequences has been achieved only on a very small scale^{10,17}, insufficient for sequencing studies by established

	1	10	20
IF- α 1	CYS-ASP-LEU-PRO-GLU-THR-HIS-SER-LEU-ASP-ASN-ARG-ARG-THR-LEU-MET-LEU-LEU-ALA-GLN-MET-SER-ARG-ILE-		
IF- α A	CYS-ASP-LEU-PRO-GLN-THR-HIS-SER-LEU-GLY-SER-ARG-ARG-THR-LEU-MET-LEU-LEU-ALA-GLX-MET-ARG(ARG)ILE-		
IF- α B	CYS-ASP-LEU-PRO-GLN-THR-HIS-SER-LEU-GLY-ASN-ARG-ARG-THR-LEU-MET-LEU-LEU-ALA-GLN-MET-GLY-ARG-ILE- GLU ASP ALA ILE SER ARG		
	30	40	
IF- α 1	SER-PRO-SER-SER-CYS-LEU-MET-ASP-ARG-HIS-ASP-PHE-GLY-PHE-PRO-GLN-GLU-GLU-PHE-ASP-GLY-ASN-GLN-PHE-		
IF- α A	SER-LEU-PHE-SER-CYS-LEU-LYS-ASP-ARG-HIS-ASP-PHE-GLY-PHE-PRO-GLN-GLU-GLU-PHE- — -GLY-ASN-GLN-PHE- ARG		
IF- α B	SER-LEU-PHE-SER-CYS-LEU-LYS-ASP-ARG-HIS-ASP-PHE-GLY-PHE-PRO-GLN-GLU-GLU-PHE-ASP-GLY-ASN-GLN-PHE- PRO SER MET HIS ARG ARG ILE		
	50	60	70
IF- α 1	GLN-LYS-ALA-PRO-ALA-ILE-SER-VAL-LEU-HIS-GLU-LEU-ILE-GLN-GLN-ILE-PHE-ASN-LEU-PHE-THR-THR-LYS-ASP-		
IF- α A	GLN-LYS)ALA-GLU-ALA-ILE-SER-VAL-LEU-HIS-GLU-MET-ILE-GLN-GLN-ILE-PHE-ASN-LEU-PHE-SER-THR-LYS-ASP- THR PRO		
IF- α B	GLN-LYS-ALA-GLN-ALA-ILE-SER-VAL-LEU-HIS-GLU-MET-ILE-GLN-GLN-THR-PHE-ASN-LEU-PHE-SER-THR-LYS-ASP- THR PRO LEU THR GLX		
	80	90	
IF- α 1	SER-SER-ALA-ALA-TRP-ASP-GLU-ASP-LEU-LEU-ASP-LYS-PHE-CYS-THR-GLU-LEU-GLN-GLN-LEU-ASN-ASP-LEU-		
IF- α A	SER-SER-ALA-ALA-GLX-ASP-GLU-THR-LEU-LEU-ASP-ASP-PHE-TYR-THR-PRO-LEU-TYR-		
IF- α B	SER-SER-ALA-ALA-TRP-ASP-ASP-ASP-LEU-LEU-ASP- — -PHE-CYS-THR-GLU-LEU-TYR-GLN-GLN-LEU-ASN-ASP-LEU- THR GLX THR SER		
	100	110	120
IF- α 1	GLU-ALA-CYS-VAL-MET-GLN-GLU-GLU-ARG-VAL-GLY-GLU-THR-PRO-LEU-MET-ASN-ALA-ASP-SER-ILE-LEU-ALA-VAL-		
IF- α A		GLU-ASP-SER-ILE-LEU-ALA-VAL-	
IF- α B	GLU-ALA-CYS-VAL-MET-LEU VAL-GLY-GLU-THR-PRO-LEU-MET-ASN-ALA-ASP-SER-ILE-LEU-ALA-VAL-		
	130	140	
IF- α 1	LYS-LYS-TYR-PHE-ARG-ARG-ILE-THR-LEU-TYR-LEU-THR-GLU-LYS-LYS-TYR-SER-PRO-CYS-ALA-TRP-GLU-VAL-VAL-		
IF- α A	ARG-LYS-TYR-PHE-GLN-ARG)ILE-THR-LEU-TYR-LEU-LYS)GLU-LYS-LYS)TYR-SER-PRO-CYS-ALA-TRP-GLU-VAL-VAL-		
IF- α B	ARG-LYS-TYR-PHE-GLN-ARG-ILE-THR-LEU-TYR-LEU-THR-GLU-LYS-LYS-TYR-SER-PRO-CYS-ALA-TRP-GLU-VAL-VAL- LYS ARG		
	150	160	
IF- α 1	ARG-ALA-GLU-ILE-MET-ARG-SER-LEU-SER-LEU-SER-THR-ASN-LEU-GLN-GLU-ARG-LEU-ARG-ARG-LYS-GLU		
IF- α A	ARG-ALA-GLU-ILE-MET-ARG-SER-PHE-SER-LEU-SER-THR-ASN-LEU-GLN-GLU-SER-LEU-ARG-SER-LYS-GLU		
IF- α B	ARG-ALA-GLU-ILE-MET-ARG)SER-LEU-SER-PHE-SER-THR-ASN-LEU-GLN-GLU-ARG)LEU-ARG)ARG-LYS-GLU PHE LEU LYS SER ASX		

Fig. 2 Summary of amino acid sequences identified in Namalwa interferon. The sequences determined in tryptic (and some other) peptides of IFN- α A and tryptic and chymotryptic peptides of IFN- α B are aligned by homology with the complete sequence of leukocyte IFN 1 (IFN- α 1) determined by analysis of cDNA (ref. 26). The sequences are written in the standard three-letter notation. In addition, *Ile* denotes Ile or, less likely, Leu; *Leu* denotes Leu or, less likely, Ile (the method of Chang *et al.*²⁴ does not clearly distinguish between these residues); and *Arg* denotes Arg or Lys (residue 23 of IFN- α A provides a site for tryptic cleavage, and either arginine or lysine could be placed in this position). The numbering system is that of IFN- α 1; IFN- α A has a deletion at residue 44 (—). Peptide sequences which have not been shown experimentally to be connected are separated by parentheses. Gaps in the sequences indicate positions for which no residue has been identified. Where more than one residue has been identified at a single position, the alternative residues are written below the line.

methods. Complete sequences of further individual components may well be obtained more readily from the analysis of nucleotide sequences in cloned cDNA, and, indeed, a second species of leukocyte IFN cDNA has recently been detected (M. Streuli, S. Nagata and C. Weissmann, unpublished results quoted in ref. 4).

The sequence of IFN- α A differs from that described in ref. 26 in at least 20 positions, whereas peptides in IFN- α B were, on the whole, more closely related to the published sequence. However, Leu 60 and Ile 64 have not been found in peptides isolated from IFN- α B. The analysis of peptide sequences is not yet complete, so the sequence of Mantei *et al.*²⁶ may be present in purified Namalwa IFN, but probably only as a minor component.

If the sequence of Mantei *et al.* is included, multiple residues have been identified at 41 positions out of 166 (25%). Although this is not the total figure for mismatch, clearly the several leukocyte-type IFNs are much more closely related than are

leukocyte and fibroblast IFNs, between which 71% of the amino acid residues differ⁴. A quantitative study of homology is not yet justified, as the data base is incomplete, but note that a highly conserved region, residues 133–151, where no differences have been detected between the various leukocyte-type IFNs, is also a region which shows strong homology between fibroblast and leukocyte IFNs⁴. This suggests a common functional role for this portion of the polypeptide chain.

The low content of aminosugars and the lack of detection of typical glycopeptides (characterized by a lower elution volume from Sephadex G-50 columns and a lower R_F on TLC than would be expected from their amino acid compositions) suggest that the major components of lymphoblastoid IFN are not glycosylated. Also, no sequence of the type Asn-X-(Ser or Thr), generally required for glycosylation of asparagine residues, has been identified. A glycosylation site could, however, be present in residues 91–113 of IFN- α A, which have not been placed in sequence. The neutral sugar content has not been deter-

mined, so some O-linked glycosylation, or glycosylation of minor components, cannot be excluded. Several observations^{7,9,15,18,19,27} suggest that leukocyte and lymphoblastoid IFNs are glycosylated, although some workers have doubted that leukocyte IFN is a glycoprotein^{12,28}. Further study is required before a definite conclusion can be reached.

The total number of different polypeptide sequences constituting human leukocyte IFN (and hence the number of structural genes) may be sufficient to account for all the heterogeneity seen by isoelectric focusing and gel electrophoresis (at least seven components were detected here, as shown in Fig. 1, but it has not been demonstrated that all of these are active as IFNs). If so, the different apparent molecular weights of the different components of IFN as determined by SDS-gel electrophoresis are not related to significant differences in the true molecular weights, but rather to such factors as binding of different amounts of SDS or different conformations of IFN-SDS complexes. The separation by gel filtration into IFN- α A and IFN- α B is also probably not related to significant differences in molecular weights, but to different conformations, IFN- α A being, presumably, more elongated in structure.

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Importance of multiple interferon structural genes

The functional significance of multiple structural genes for IFN remains to be elucidated. Multiple genes have been identified for other proteins, such as actin^{29–31}. With actin, the proteins are synthesized in large amounts and there are well established differences in functional properties between the different gene products. Possibly, different IFN gene products have different biological activities, or different specificities towards different target cells, and they may be differentially induced by different viruses.

We thank Dr C. Weissmann for making available sequence information before publication.

Note added in proof: The complete sequence of a second species of human leukocyte interferon (IFN- α 2), deduced from the nucleotide sequence of cloned cDNA, has recently been determined³³. IFN- α 2 is very similar to, and may be identical with, one of the components of IFN- α A.

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Human leukocyte interferon produced by *E. coli* is biologically active

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A human leukocyte interferon cDNA was enzymatically synthesized, inserted into the vector pBR322, and cloned in Escherichia coli. The DNA sequence codes for a 23-amino acid signal peptide followed by an interferon polypeptide of 165 amino acids. An expression plasmid was constructed which permits the synthesis in E. coli of 2.5×10^8 units of interferon per litre of culture. This LeIF protected squirrel monkeys from lethal encephalomyocarditis virus infection.

THE interferons (IFNs) are a family of proteins characterized by a potent ability to confer a virus-resistant state in their target cells^{1,2}. In addition, IFNs can inhibit cell proliferation and modulate immune response (reviewed in ref. 2). These properties have led to the clinical use of IFN for the treatment of viral infections and malignancies.

The classical, or type I, IFNs consist of at least two distinct gene products which differ antigenically^{3,4} and have different target cell specificities⁵. Leukocyte interferon (LeIF) and fibro-

blast interferon (FIF) are named for the cells from which they are derived. Messenger RNAs for LeIF and FIF have been isolated and translated *in vitro* and in *Xenopus* oocytes^{6–8}.

Both LeIF^{9,10} and FIF¹¹ have been purified to homogeneity; reported molecular weights range from 17,500 to 21,000. The specific activities of these preparations are remarkably high, 2×10^8 to 1×10^9 U per mg protein. Unfortunately the yields from eukaryotic cells have been correspondingly low. Two litres of human blood are required to produce approximately 1 μ g of

purified LeIF⁹. Nevertheless, advances in protein sequencing techniques have permitted the determination of partial amino acid sequences¹¹⁻¹³.

Recombinant DNA technology provides an alternative method of producing large quantities of IFN using bacterial cells. Human FIF cDNA has recently been cloned^{14,15} and biologically active mature FIF expressed in *Escherichia coli*¹⁵. Likewise, a species of human LeIF cDNA has been cloned in *E. coli* and expressed in an apparent precursor form with a signal peptide attached¹⁶. We describe here the construction of a plasmid directing the high level expression in *E. coli* of a human LeIF which exhibits *in vivo* antiviral activity.

Construction and identification of clones containing LeIF cDNA sequences

Cells from the human myeloblastoid line KG-1 (ref. 17) were collected 5 h after induction with Sendai virus and the 12S sucrose gradient fraction of poly(A) RNA was isolated as described elsewhere¹⁸. This mRNA had an IFN titre of 8,000 U μg^{-1} in the *Xenopus laevis* oocyte assay⁴. Five μg of mRNA was used to prepare double-stranded cDNA by standard procedures^{19,20}. The cDNA was electrophoresed on a 6% polyacrylamide gel and 110 ng of material from 600 to 1,300 base pairs in length were recovered by electroelution. A 20-ng portion of this cDNA was tailed with deoxycytidine residues²¹, annealed with 100 ng of plasmid pBR322 (ref. 22) which had been tailed with deoxyG residues at the *Pst*I site, and used to transform *E. coli* K12 strain 294 (ref. 23). Approximately 1,000 tetracycline-resistant, ampicillin-sensitive transformants were obtained per ng of cDNA. A rapid plasmid isolation procedure²⁴ was used to prepare about 1 μg of plasmid DNA from each of 500 individual transformants. Each DNA sample was denatured and aliquots were applied to each of three nitrocellulose filters following a published procedure²⁵.

The amino acid sequences of several tryptic fragments of human LeIF have been determined (W. Levy, J. Shively and S. P., unpublished results). This information permitted the design of synthetic deoxyoligonucleotides potentially complementary to different regions of LeIF mRNA. The two tryptic peptides T-1 and T-13 were selected because they had amino acid sequences requiring the synthesis of only 12 and four undecamers, respectively, to account for all possible coding sequences (Fig. 1). Four sets of deoxyoligonucleotide probes were synthesized for each sequence, containing either three (T-1A, B, C, D) or one (T-13A, B, C, D) oligonucleotides each.

	Tryptic peptide (T-1)	Tryptic peptide (T-13)
Protein	Ala-Glu-Ile-Met-Arg	---His-Glu-Met-Ile-Gln---
mRNA	5' GCN GA ^A AUC ^A AUG ^A C ^A GN	5' CA ^C GA ^A AUG ^A AUC ^A CA ^A G
Complementary DNA primers	3' CTT A ^T TAC GC (T-1A) ---C----- (T-1B) -----T- (T-1C) ---C-----T- (T-1D)	3' GTA CTT TAC TA (T-13A) ---G----- (T-13B) -----C----- (T-13C) ---G-----C----- (T-13D)

Fig. 1 Synthetic deoxyoligonucleotides designed to prime cDNA synthesis from LeIF mRNA. Amino acid sequences are given for peptide 1 and a portion of peptide 13 derived from a tryptic digest of human LeIF- β 1 (W. Levy, J. Shively and S. P., unpublished results). All potential mRNA sequences coding for these peptides are shown. The indicated complementary deoxyoligonucleotides 11-bases long were chemically synthesized by the phosphotriester method³⁶. Four individual primers were prepared in the T-13 series. The 12 T-1 primers were prepared in four pools of three primers each using a strategy described elsewhere¹⁵. ³²P-labelled cDNA was prepared from these primers using published reaction conditions³⁷. The reactions (60 μl) were performed in 20mM Tris-HCl (pH 8.3), 20mM KCl, 8mM MgCl₂, 30mM β -mercaptoethanol. Reactions included one μg of each primer (that is 12 μg total for T-1 series, 4 μg total for T-13 series), two μg of 12S fraction mRNA from induced cells (or 10 μg of poly(A) mRNA from uninduced cells), 0.5 mM dATP, dGTP, dTTP, 200 μCi [α -³²P]dCTP (2-3,000 Ci mmol⁻¹; Amersham), and 60 U reverse transcriptase. Product was separated from unincorporated label by gel filtration on a 10-ml Sephadex G-50 column, treated with 0.3 M NaOH for 30 min at 70°C to destroy RNA, and neutralized with HCl. Hybridizations were performed as described^{15,25}.

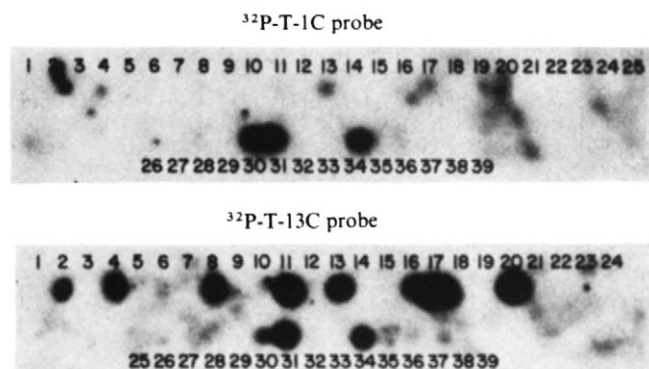


Fig. 2 Hybridization of potential LeIF cDNA recombinant plasmids with ³²P-labelled synthetic deoxyoligonucleotides. Plasmid DNA from the 39 clones was prepared by a standard cleared lysate procedure³⁸ and purified by Biorad Agarose A-50 column chromatography. Samples (3 μg) of each preparation were linearized by treatment with *Eco*RI, denatured in alkali and spotted on two separate nitrocellulose filters (1.5 μg per spot)²⁵. Individual synthetic deoxyoligonucleotide primers and primer pools were phosphorylated with [γ -³²P]ATP as follows: 50 pmol of oligonucleotide and 100 pmol of [γ -³²P]ATP (2,500 Ci mmol⁻¹; New England Nuclear) were combined in 30 μl of 60 mM Tris-HCl (pH 8) 10 mM MgCl₂, 15 mM β -mercaptoethanol. Two units of T4 polynucleotide kinase were added and, after 30 min at 37°C, ³²P-labelled primers were purified by chromatography on 10-ml Sephadex G-50 columns. Hybridizations were performed using 10⁶ c.p.m. of primer T-13C or 3 $\times$ 10⁶ c.p.m. of primer pool T-1C. The hybridizations were performed at 15°C for 14 h in 6 $\times$ SSC, 10 $\times$ Denhardt's solution, as described by Wallace *et al.*²⁸. Filters were washed for 5 min (three times) at 0°C in 6 $\times$ SSC, dried, and exposed to X-ray film. Results are shown above for primer T-13C and primer pool T-1C.

These synthetic deoxyoligonucleotides were used to prime the synthesis of radiolabelled single-stranded cDNA for use as hybridization probes (see Fig. 1 legend). The template mRNA was either the 12S RNA from KG-1 cells induced with Sendai virus (8,000 U IFN activity μg^{-1}) or total poly(A) mRNA from uninduced leukocytes (<10 U IFN activity μg^{-1}).

The three sets of nitrocellulose filters containing the 500 plasmid samples were hybridized with three different cDNA probes: (1) induced cDNA primed with the T-1 set of primers, (2) T-13-primed induced cDNA, and (3) uninduced cDNA prepared by using both sets of primers. Clones were considered positive if they hybridized more strongly to one or both of the induced cDNA probes (1) and (2) than to the total uninduced probe(3). Thirty clones (pL1-pL30) were selected from the 500 for further analysis.

At this time a partial-length (~750-base pair) LeIF cDNA recombinant plasmid pIFL104, as identified by a mRNA hybridization selection procedure, became available¹⁸. A unique 260-base pair *Bgl*/II restriction fragment isolated from this clone was labelled with ³²P (ref. 26) and used as probe to screen another 400 transformants by an *in situ* colony screening procedure²⁷. Nine colonies (pL31-pL39) were identified which hybridized to different extents with this probe. Plasmid DNA was prepared from all 39 potential LeIF cDNA clones and rescreened with the same 260-base pair DNA probe using the dot hybridization procedure²⁵. Three plasmids (pL4, pL31, pL34) exhibited very strong hybridization signals, four (pL13, pL30, pL32, pL36) hybridized moderately, and three (pL6, pL8, pL14) hybridized weakly with the probe.

The 39 potential LeIF cDNA recombinant plasmids were also screened with ³²P-labelled synthetic undecamers (individual T-1 primer pools or individual T-13 primers) directly as hybridization probes. The hybridization conditions were chosen such that perfect base pairing should be required for detectable hybridization signals²⁸. Plasmid pIFL104 was found to give significant hybridization with primer pool T-1C and primer T-13C, but no detectable hybridization with the other undecamers. As shown in Fig. 2, several of the 39 potential LeIF plasmids (pL2, 4, 13, 17, 20, 30, 31, 34) also hybridized with both of these probes. Restriction analysis revealed that only one of these plasmids, pL31, also contained a 260-base pair internal *Bgl*/II fragment.

*Pst*I digestion of pL31 showed the size of the cDNA insert to be approximately 1,000 base pairs.

DNA sequence of recombinant plasmid pL31

The entire *Pst*I insert of pL31 was sequenced by both the Maxam-Gilbert chemical method²⁹ and an enzymatic method³⁰ using the bacteriophage M13 cloning vector mp7 (P. S., unpublished results). The DNA sequence is shown in Fig. 3. Protein sequence information from LeIF (ref. 13; W. Levy, J. Shively and S. P., unpublished results) permitted the determination of the correct translational reading frame and allowed us to predict the entire LeIF type A (our term for the LeIF encoded by pL31) amino acid sequence, including a precursor segment or signal peptide. The first ATG translational initiation codon is found 60 nucleotides from the 5' end of the sequence and is followed, 188 codons later, by a TGA termination triplet; there are 335 untranslated nucleotides at the 3' end, followed by a poly(A) sequence. The putative signal peptide (presumably involved in the secretion of mature LeIF from leukocytes) is 23 amino acids long. The 165-amino acid polypeptide constituting the mature LeIF has a calculated molecular weight of 19,390. The DNA sequence also shows that the tryptic peptides T1 and T13 of LeIF β_1 (Fig. 1) correspond to amino acids 145–149 and 57–61, respectively, of LeIF A. The actual DNA coding sequences found in these two regions are those represented by primer pool T-1C and primer T-13C, as the hybridization data in Fig. 2 had suggested.

The amino acid sequence deduced from the DNA sequence of LeIF type A has only partial identity with the directly determined NH₂-terminal amino acid sequences of LeIF α 1 (20 out of 22 positions)¹³ and human lymphoblastoid IFN (16 out of 20 positions)¹². It also differs significantly from the complete amino acid sequence recently deduced from the nucleotide sequence of LeIF I (ref. 31). LeIF I codes for a mature IFN of 166 amino acids, as compared to 165 amino acids for LeIF A. The extra amino acid (aspartic acid) of LeIF I is between amino acids 43 and 44 of LeIF A. Furthermore, only 137 of the remaining 165 amino

acids (83%) are identical. The single longest stretch of homology between the two LeIFs is 19 amino acids (residues 132–150), and in one region (residues 100–106) only two out of seven amino acids are identical. The amino acid homology in the signal peptide sequence is 74% (17/23 identical residues). The glycine-cysteine cleavage site between the signal and IFN sequence is the same in both IFNs. In addition, the 3' untranslated regions of LeIF A and LeIF I mRNAs (335 compared with 242 nucleotides) differ considerably. The explanation for these discrepancies lies in the fact that there is a family of non-identical LeIF genes (including pL4, pL13, pL30, pL32), evidence for which will be reported soon.

Expression of leukocyte pre-interferon A

The precursor form of LeIF A (Le-preIF A) was expressed in *E. coli* by ligating the 1,000-base pair *Pst*I insert into the *Pst*I site of the plasmid pNCV. pNCV is a derivative of pBR322 containing part of the *E. coli* tryptophan operon (extending from the promoter-operator region through the *trp* E gene, with a deletion³² of a portion of the *trp* leader and *trp* E coding regions) which has been modified to contain a unique *Pst*I site at the C-terminal end of the *trp* E protein coding sequence (H. H., unpublished results). The LeIF A DNA sequence can be inserted into pNCV in either of two orientations. As shown in Table 1, only plasmids having inserts which are orientated correctly with respect to the direction of transcription from the *trp* promoter give detectable IFN activity. Extracts of *E. coli* containing the plasmid pLe-preIF A2 gave approximately 480,000 U of activity per litre of culture, considerably higher than the maximal 20,000 U l⁻¹ reported by Nagata *et al.* for Le-preIF D (ref. 16). The Le-preIF A produced by *E. coli* can be visualized by staining and co-migrates with human growth hormone (191 amino acids; molecular weight 22,000) on SDS-polyacrylamide gels (J. Perry, unpublished results). This suggests that the expression of Le-preIF A in pNCV is due to translation initiating at the signal peptide ATG start codon.

Fig. 3 Nucleotide and amino sequences of pL31 (LeIF A). The DNA sequences of both strands of the *Pst*I insert were determined by the Maxam-Gilbert technique and by the dideoxy chain termination procedure³⁰ after subcloning *Sau*3a fragments of pL31 into the M13 vector mp7 (P. S., unpublished results). Numbers above each line refer to amino acid position (S refers to signal peptide) and numbers below each line to nucleotide position.

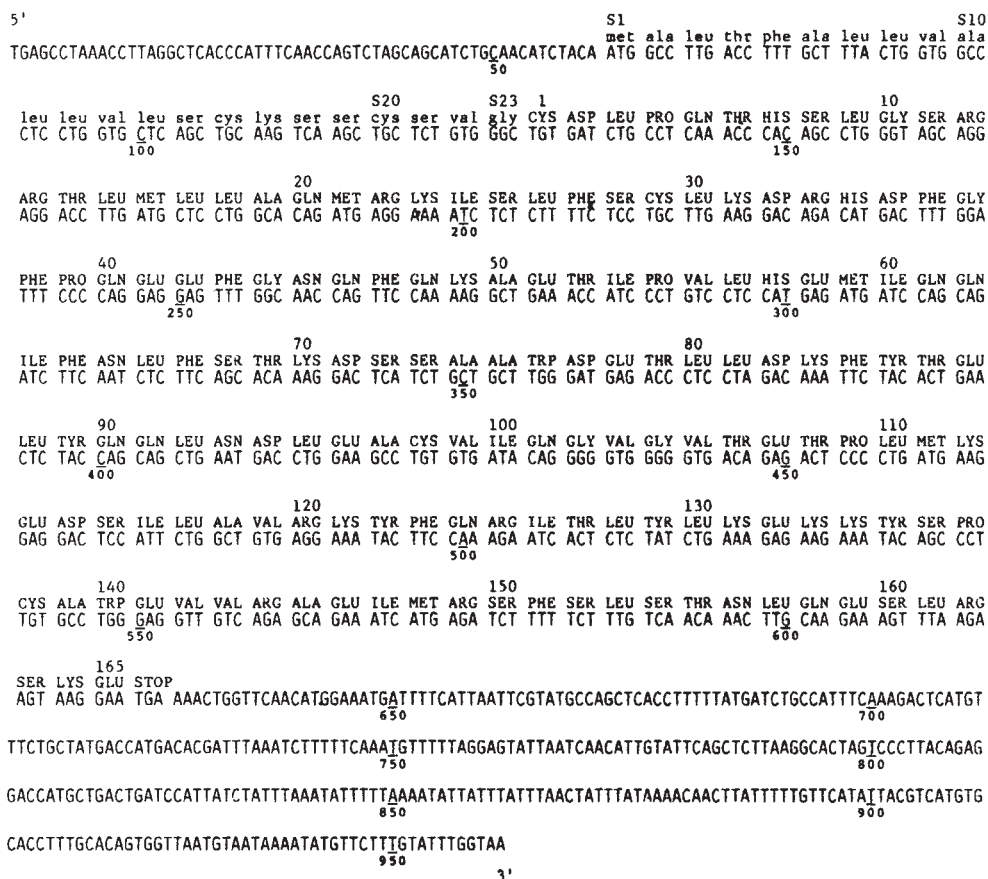


Table 1 Interferon activity in extracts of *E. coli*

<i>E. coli</i> 294 transformed by:	Cell density (cells ml ⁻¹)	IFN activity (U per ml culture)	LeIF molecules per cell
pLe-preIF A2	3.5 × 10 ⁸	480	120
pLe-preIF A3	3.5 × 10 ⁸	0	0
pLeIF A25	3.5 × 10 ⁸	36,000	9,000
pLeIF A25	1.8 × 10 ⁹	250,000	12,000
pLeIF A118	3.5 × 10 ⁸	20	5

The 1,000-base pair *Pst*I insert (0.5 µg) from pL31 was ligated to 0.2 µg of *Pst*I-cleaved pNCV and the product used to transform *E. coli* 294. Recombinant plasmids containing inserted LeIF cDNA were identified by colony hybridization²⁷ using the ³²P-labelled 260-base pair *Bgl*II fragment from pL31 as a hybridization probe. Digestion of these plasmids with *Pvu*II identified clones with the LeIF A DNA sequence orientated in the correct orientation (pLe-preIF A2) and in the opposite orientation (pLe-preIF A3). The construction of pLeIF A25 and pLeIF A118 for direct expression of LeIF A is described in Fig. 4 legend. pLeIF A118 has the *trp* promoter orientated such that transcription proceeds away from the LeIF A gene. Extracts were prepared for IFN assay as follows. One-ml cultures were grown in L-broth containing 5 µg ml⁻¹ tetracycline to an A₅₅₀ of ~1.0, then diluted into 25 ml of M9 medium containing 0.2% glucose, 0.5% casamino acids and 5 µg ml⁻¹ tetracycline. 10-ml samples were collected by centrifugation when A₅₅₀ reached 1.0 and cell pellets were suspended in 1 ml of 15% sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA. One mg of lysozyme was added and, after 5 min at 0 °C, cells were disrupted by sonication. The samples were centrifuged for 10 min at 15,000 r.p.m. in a Sorvall SM-24 rotor. IFN activity in the supernatants was determined by comparison with LeIF standards by the cytopathic effect (CPE) inhibition assay². To determine the number of IFN molecules per cell, a LeIF specific activity of 4 × 10⁸ U mg⁻¹ was used⁹. To measure pH2 stability, the 250,000 U ml⁻¹ extract of *E. coli* 294/pLeIF A25 was diluted 500-fold with MEM giving a concentration of 500 U ml⁻¹. A LIF standard (Wadley Institute) previously titrated against the NIH LIF standard, was also diluted to a final concentration of 500 U ml⁻¹. Aliquots (1 ml) were adjusted to pH 2 with 1 M HCl, incubated at 4 °C for 52 h, neutralized by addition of NaOH and IFN activity determined by the standard CPE inhibition assay². To measure neutralization by antibody, 25-µl aliquots of the 500 U ml⁻¹ samples (untreated) were incubated with 25 µl of rabbit anti-human LeIF (10,000 neutralizing units ml⁻¹) for 60 min at 37 °C, centrifuged at 12,000g for 5 min and the supernatant assayed.

Direct expression of mature leukocyte interferon

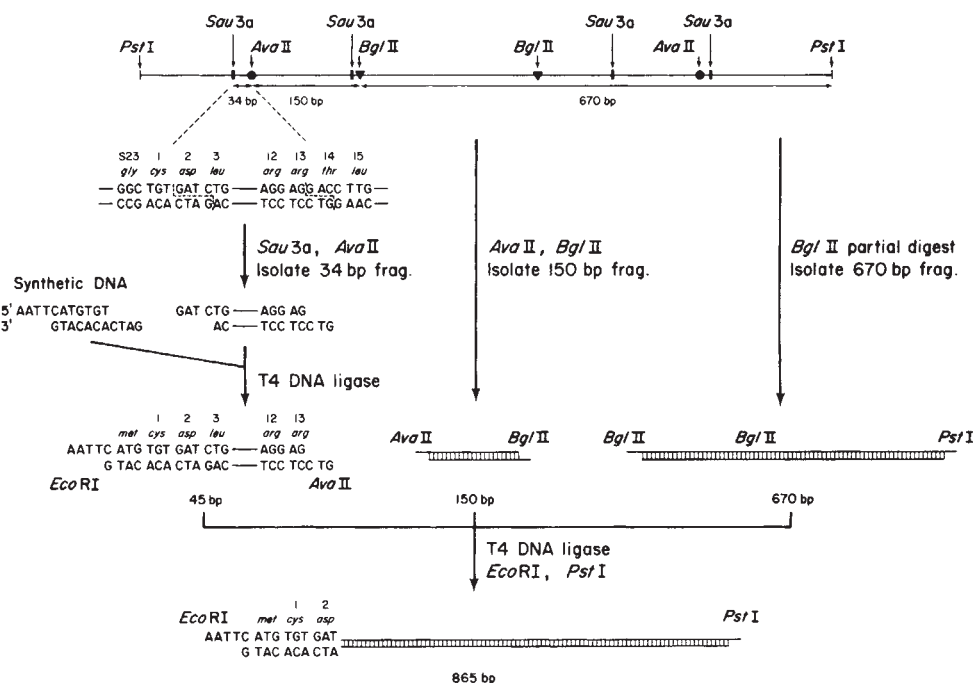
The procedure followed to express LeIF A directly as a mature IFN polypeptide was a variation of that employed previously for human growth hormone²⁰. As shown in Fig. 4, a *Sau*3a restriction endonuclease site is conveniently located between codons 1 and 2 of the LeIF A sequence. Two synthetic deoxyoligonucleotides were designed which incorporate an ATG translational initiation codon, restore the codon for amino acid 1 (cysteine), and create an *Eco*RI sticky end. These oligomers were ligated to a 34-base pair, *Sau*3a–*Ava*II fragment of pL31. The resulting 45-base pair product was ligated to two additional DNA fragments to construct an 865-base pair synthetic–natural hybrid gene which codes for LeIF A and which is bounded by *Eco*RI and *Pst*I restriction sites. This gene was inserted into pBR322 between the *Eco*RI and *Pst*I sites to give the plasmid pLeIF A1.

Next, a 300-base pair *Eco*RI fragment was constructed which contains the *E. coli trp* promoter, operator, and the *trp* leader ribosome binding site but stops short of the ATG sequence for initiation of translation of the leader peptide (D. Kleid and D. Yansura, unpublished results). This DNA fragment was cloned into the *Eco*RI site of pLeIF A1. Transformants carrying plasmids with the *trp* promoter orientated in either possible orientation were assayed for IFN activity (Table 1). Clone pLeIF A25, in which the *trp* promoter was inserted in the desired orientation, gives high levels of activity (2.5 × 10⁸ U l⁻¹). Low levels of activity are obtained even if the *trp* promoter reads away from the LeIF A gene, as in the case of pLeIF A118 (2.0 × 10⁴ U l⁻¹). The IFN produced by *E. coli* 294/pLeIF A25 behaves like authentic human LeIF; it is stable to treatment at pH2 and is

Fig. 4 Construction of a gene coding for the direct synthesis of mature human LeIF A in *E. coli*. 250 µg of plasmid pL31 were digested with *Pst*I and the 1,000-base pair insert isolated by gel electrophoresis on a 6% polyacrylamide gel. Approximately 40 µg of insert were electroeluted from the gel and divided into three aliquots for further digestion.

a, A 16-µg sample of this fragment was partially digested with 40 U of *Bgl*II for 45 min at 37 °C and the reaction mixture purified on a 6% polyacrylamide gel. Approximately 2 µg of the desired 670-base pair fragment were recovered. b, Another sample (8 µg) of the 1,000-base pair *Pst*I insert was restricted with *Ava*II and *Bgl*II. One µg of the indicated 150-base pair fragment was recovered after gel electrophoresis.

c, 16 µg of the 1,000-base pair piece were treated with *Sau*3a and *Ava*II. After electrophoresis on a 10% polyacrylamide gel, approximately 0.25 µg (~10 pmol) of the 34-base pair fragment were recovered. The two indicated deoxyoligonucleotides, 5'-dAATTCATGTGT (fragment 1) and 5'-dGATCACACATG (fragment 2) were synthesized by the phosphotriester procedure³⁶. Fragment 2 was phosphorylated as follows: 200 µl (~40 pmol) of [³²P]ATP (5,000 Ci mmol⁻¹; Amersham) was dried down and resuspended in 30 µl of 60 mM Tris-HCl (pH 8), 10 mM MgCl₂, 15 mM β-mercaptoethanol, containing 100 pmol of DNA fragment and 2 U of T4 polynucleotide kinase. After 15 min at 37 °C, 1 µl of 10 mM ATP was added and the reaction allowed to proceed another 15 min. The mixture was then heated at 70 °C for 15 min, combined with 100 pmol of 5'-OH fragment 1 and 10 pmol of the 34-base pair *Sau*3a–*Ava*II fragment. Ligation was performed for 5 h at 4 °C in 50 µl of 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM ATP and 10 U T4 DNA ligase. The mixture was electrophoresed on a 6% PA gel and the 45-base pair product recovered by electroelution. 860,000 Cerenkov c.p.m. were recovered (~30 ng, 1 pmol), combined with 0.5 µg (5 pmol) of the 150-base pair *Ava*II–*Bgl*II fragment and 1 µg (2 pmol) of the 670-base pair *Bgl*II–*Pst*I fragment. The ligation was performed at 20 °C for 16 h using 20 U of T4 DNA ligase. The ligation was inactivated by heating to 65 °C for 10 min. The mixture was then digested with *Eco*RI and *Pst*I to eliminate polymers of the gene. The mixture was purified by 6% polyacrylamide gel electrophoresis. 36,000 c.p.m. (~0.04 pmol, 20 ng) of 865-base pair product were isolated. One half (10 ng) of this was ligated into pBR322 (0.3 µg) between the *Eco*RI and *Pst*I sites. Transformation of *E. coli* 294 gave rise to 70 tetracycline-resistant, ampicillin-sensitive transformants. Plasmid DNA isolated from 18 of these transformants was digested with *Eco*RI and *Pst*I. 16 of the 18 plasmids had an *Eco*RI–*Pst*I fragment 865 base pairs in length. One µg of one of these, pLeIF A1, was digested with *Eco*RI and ligated to a 300-base pair *Eco*RI fragment (0.1 µg) containing the *E. coli trp* promoter and *trp* leader ribosome binding site. Transformants containing the *trp* promoter were identified using a ³²P-*trp* probe in conjunction with the Grunstein–Hogness colony screening procedure²⁷. An asymmetrically located *Xba*I site in the *trp* fragment allowed us to determine recombinants in which the *trp* promoter was orientated in the direction of the LeIF A gene. bp, Base pair.



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Table 2 Antiviral effect of various LeIF preparations against EMC virus infection of squirrel monkeys

Treatment	Survivors	Serum PFU ml ⁻¹			
		Day 2	Day 3	Day 4	
Control	0/3	10	3 × 10 ⁴	> 10 ⁵	> 3.4 × 10 ⁴
(bacterial proteins)		0	0	1,200	
Bacterial LeIF A		0	0	0	
LeIF standard	3/3	0	0	0	
	3/3	0	0	0	
		0	0	0	
		0	0	0	

All monkeys were male (average weight 713 g) and had no EMC virus antibodies prior to infection. The monkeys were infected intramuscularly with 100 × LD₅₀ EMC virus (determined in mice). The control treated monkeys died at 134, 158 and 164 h post-infection. IFN treatments of 10⁶ U were intravenously at -4, +2, 23, 29, 48, 72, 168 and 240 h, relative to infection. The bacterial LeIF was a column chromatography fraction (R. Wetzel, unpublished results) from a lysate of *E. coli* 294/pLeIF A25 at a specific activity of 7.4 × 10⁶ U mg⁻¹ protein. The control bacterial proteins were an equivalent column fraction from a lysate of *E. coli* 294/pBR322 at twice the total protein concentration. The LeIF standard was Sendai virus-induced IFN from normal human buffy-coat cells, purified chromatographically to a specific activity of 32 × 10⁶ U per mg protein. Survival of the monkeys in the IFN-treated group is significant at the 5% level by a log rank χ^2 analysis³⁹. PFU, plaque forming unit.

neutralized by rabbit anti-human LIF antibodies. The IFN has been partially purified and has an apparent molecular weight of 20,000 (R. Wetzel and M. Ross, unpublished results).

In vivo antiviral activity of LeIF A

The *in vivo* efficacy of IFN needs the presence of macrophages and NK cells, and the *in vivo* mode of action seems to involve stimulation of these cells³³⁻³⁵. Thus, it remained possible that the IFN produced by *E. coli* 294/pLeIF A25, though having antiviral activity in the cell culture assay, would not be active in infected animals. Moreover, the *in vivo* antiviral activity of the bacterially produced, non-glycosylated LeIF A might be different from the IFN derived from human 'buffy coat' leukocytes. Therefore the biological activity of bacterially synthesized LeIF A (~2% pure) was compared with buffy coat LeIF (~8% pure) in lethal encephalomyocarditis (EMC) virus infection of squirrel monkeys (Table 2). The control monkeys showed progressive lethargy, loss of balance, flaccid paralysis of the hind limbs and watering of the eyes commencing ~8 h before death. The IFN-treated monkeys showed none of these signs; they remained active at all times and developed no viremia (Table 2). The one monkey in the control group which did not develop viremia by 4 days died latest (164 h post-infection) but showed high titres of virus in the heart and brain on post mortem. The IFN-treated monkeys did not develop antibodies to EMC virus as determined 14 and 21 days after infection. These results demonstrate that the antiviral effects of LeIF preparations in the infected animals can be attributed solely to IFN because the contaminating proteins are quite different in the bacterial and buffy coat preparations. In addition, these results indicate that glycosylation is not required for the *in vivo* antiviral activity of LeIF A. The pharmacokinetics and toxic effects of the natural and bacterially produced LeIFs are currently being compared.

Discussion

With 12S poly(A) mRNA from induced human leukocytes, a full length LeIF A cDNA has been synthesized and cloned in the *E. coli* vector pBR322. This particular clone (pL31) was identified by colony screening using a DNA fragment from a shorter LeIF cDNA clone¹⁸ as hybridization probe. pL31 was also found to hybridize to synthetic deoxyoligonucleotide probes whose sequences were based upon LeIF amino acid sequence data. Several additional types of LeIF recombinant plasmids were identified by hybridization to radiolabelled LeIF cDNA probes. These probes were prepared by using the synthetic deoxyoligonucleotides as primers for the synthesis of cDNA using 12S poly(A) mRNA from induced cells as template. This approach should be generally applicable for the

identification of recombinant plasmids obtained through cDNA cloning of rare mRNA species, assuming that some protein sequence information is available.

The 961-base pair DNA sequence of pL31 codes for 188 amino acids, 23 of which are probably cleaved during the process of secretion of mature LeIF from leukocytes. The 188-amino acid leukocyte pre-IF A was expressed in *E. coli* under *trp* promoter control and bacterial extracts were found to be biologically active in tissue culture assay. It is not yet known if the IFN activity is associated with the pre-interferon itself, or if a small proportion of Le-preIF A is processed by *E. coli* to a biologically active form. A plasmid was also constructed which codes for high level expression of the 165 amino acid LeIF A preceded only by a methionine. The present yield of 2.5 × 10⁸ U per litre of culture corresponds to about 600 µg of IFN per litre of culture, assuming an LeIF specific activity of 4 × 10⁸ U mg⁻¹ (ref. 9).

This bacterially synthesized LeIF A exhibits the pH 2 stability and immunological characteristics of authentic human LeIF, and it protects squirrel monkeys against lethal infection with EMC virus when treatments are initiated prior to viral challenge. Studies are under way to determine if bacterially produced LeIF A has therapeutic, as well as prophylactic, value. Additionally, the *in vivo* activity of LeIF A is being compared with the activities of bacterially produced human fibroblast IFN¹⁵, and several other types of bacterially expressed LeIF.

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LETTERS

Morphology of the triple QSO PG1115+08

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Weymann *et al.*¹ have reported that two faint unresolved objects located within 3 arcs of the QSO PG1115+08 have also the same spectra. The emission lines of C III λ 1,909 and C IV λ 1,549 at a redshift of 1.72 have central wavelengths and profiles that are indistinguishable between the objects. It is inferred that the three objects are likely to be images of the same QSO produced by intervening matter acting as a gravitational lens. Only one other example of such an effect is known, that is the double QSO 0957+56A,B. In this case, the two QSO images are of roughly equal magnitude ($V \sim 17$) and separated by 6 arcs. A plate obtained in excellent seeing by Stockton² shows an intervening galaxy at $z = 0.4$ lying about 1 arc s from one of the QSO images. The present observations were undertaken to search for evidence of an intervening galaxy in PG1115+08, appearing either directly in a deep image, or as a difference in colour between components, and also to look for any structure in the three images. A colour difference could arise, as was the case for the double QSO, if a galaxy is nearly coincident with one of the QSO images.

The Multi-Mirror Telescope (MMT) was used for this study because of its consistently excellent seeing, with images of 1 arc s or better for much of its use in the past year. As part of a study of the short-term motion and stabilization of the six images relative to each other, and of interferometry between different mirrors, we have been using Steward's intensified television camera on the MMT. Having very high gain, provided by a plumbicon television camera coupled to a four-stage image intensifier, it is ideally suited for this study of the QSO.

While the present conditions allow six images of the MMT to be co-aligned well enough to resolve the QSO components in the combined image, as was done when the first spectra of the fainter components were obtained, the image quality in good seeing is degraded. For the present work we used only one of the six 1.8-m mirrors. The camera was located directly at the Cassegrain focus, where the plate scale is $3.6 \text{ arc s mm}^{-1}$. The camera output (in standard video format with 30 full frames of 480 lines per s) was recorded on video tape, with a frame number code added to the video signal. Recordings of ~ 10 min duration were made through filters corresponding approximately to the *B*, *V* and *I* photometric bands as detailed in Table 1. The telescope was not guided during these recordings. Short integrated images were obtained off-line by first digitizing the frame and co-adding a field of $10 \times 10 \text{ arc s}$ from 30 successive frames (1 s intervals). Between 50 and 100 of these short integrations were then further co-added after being laterally translated so that the peak of the brightest QSO image was always in the same pixel. The fixed pattern of a digitized blank video field was subtracted from each 1-s video integration before co-adding. In effect, this is like precise guiding at 1-s intervals. The resulting images for the

three colour bands and also for a broad band with no filter are shown in Fig. 1. For photographic processing, the fourth root of image intensity is mapped on the linear grey scale.

The camera was mounted with the elevation axis vertical. Effects of field rotation of the altazimuth telescope are negligible over the short observing intervals. Figure 2 shows an 8-s integration in the broad band, with no centering, over a wider field including a neighbouring object, with celestial coordinates marked. Table 1 summarizes the magnitude differences and separations that are derived from analysis of the three QSO components, labelled A, B and C after Weymann *et al.*¹. The uncertainties in the mean intensity ratios expressed in stellar magnitude differences, reflected by statistical scatter in the ratios measured, are commensurate with the uncertainty in the flat-field response ($\leq 5\%$ r.m.s.) of the four stages of fibre-optic coupled Varo image intensifiers.

Because of the low declination and early transit of the object at the time of observation, these images were taken at large zenith distances. Elongation due to atmospheric dispersion can be seen in the expected vertical direction in all three images in the broadband exposure ($4,100\text{--}8,500 \text{ \AA}$), shown in Fig. 1c at $\sec z = 1.5$. The best resolution with negligible degradation due to dispersion is found in the red exposure, Fig. 1d, despite the greater zenith distance, $\sec z = 2.5$ at the time of exposure. The image profiles for components B and C show a FWHM of 0.6 arc s, and are round. The image of the bright component A is, however, distinctly not round, being apparently elongated by $\sim 0.5 \text{ arc s}$ at a position angle of about 20° . We believe that we can rule out the elongation being an artefact of the optics or data reduction, as the centering was done on the A image, and the B

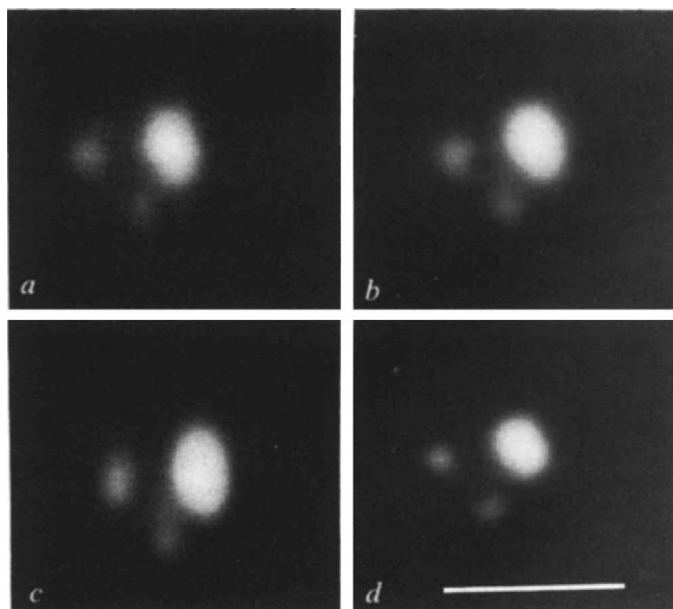


Fig. 1 Single-mirror images of PG1115+08. *a*, Blue; *b*, *V*; *c*, unfiltered; *d*, red. See Table 1 for definition of bandpasses. The images are slightly anamorphic with 13% stretching in the vertical direction. The bar gives the average scale for 5 arc s

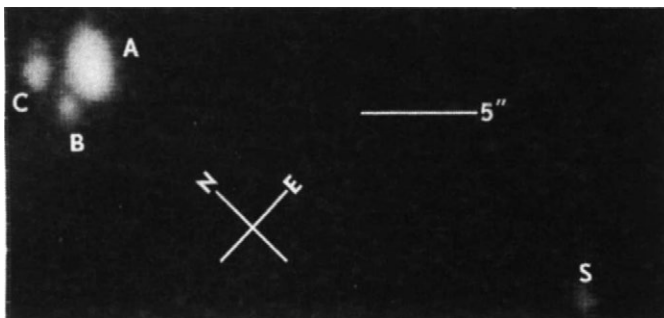


Fig. 2 A wider field image of PG1115+08 in which the celestial coordinates are marked and the components are identified. The object S is at 161° , 23 arc s from component A. The scale and distortion are the same as for Fig. 1.

and C images are indeed round. We have further data at higher magnification which may be able to yield confirmation by the techniques of speckle interferometry.

From the images and analysis above we can make the following observations.

- (1) The two companion images each appear like unresolved point sources, seeing limited at the resolution of 0.6 arc s FWHM. The primary image A is not a single stellar object. It would be consistent with two point sources about 0.5 s apart.
- (2) The data are consistent with all objects having the same colour, although there is a trend suggesting that C, the further and brighter companion, may be 0.25 mag redder in *B-I* colour.
- (3) No other nearby objects brighter than $V \sim 21$ are detected within a radius of 5 arc s from A. If there is an intervening galaxy it must either be fainter than this limit or lying well within 1 arc s of one of the components.

From the observed relative magnitudes and the fluxes measured by R. F. Green and M. Schmidt (personal communication), we can compute fluxes for each of the components. If the slight excess red flux in component C is real and were due to a galaxy similar to the one Young *et al.*³ measured in 0957+561, we can estimate the galaxy's magnitude by fitting the flux of C by a linear combination of a similar galaxy spectrum and the measured spectrum of A. The best fit is obtained with 15% of A and 17% of such a red galaxy. When the fluxes are recomputed using $C = 0.15 A$, an estimate of the galaxy flux results which suggests a somewhat fainter and slightly redder galaxy of $m_v \sim 20.7$. However, we must emphasize that colour differences are not statistically significant, and such a faint galaxy is consistent with but not required by the data.

The research reported here used the Multiple Mirror Telescope Observatory, a joint facility of the University of Arizona and the Smithsonian Institution. We thank J. T. Williams and L.

Table 1 Magnitude differences and separations of the QSO components A, B and C

Pair	Magnitude difference*				Separation (arc s)	Angle†
	<i>B</i> †	<i>V</i> ‡	No filter§	<i>I</i>		
A-B	2.35	2.34	2.40	2.38	1.9 ± 0.1	$263^\circ \pm 5^\circ$
A-C	2.00	1.93	1.89	1.78	2.4 ± 0.1	$317^\circ \pm 5^\circ$
B-C	0.35	0.41	0.51	0.60	2.0 ± 0.1	

* Errors are estimated to be 0.1 mag for A-B and A-C differences, and 0.2 mag for B-C.

† Blue band defined by Hoya B-390 and photocathode cutoff, $\sim 4,100$ – $4,800$ Å.

‡ Standard Johnson *V* band.

§ Half-power points with no filter are 4,100–7,600 Å.

|| Infrared band defined by Schott RG 715 filter and photocathode cutoff, $\sim 7,150$ – $8,500$ Å.

¶ Position angles calibrated assuming 161° for position angle of object marked S in Weymann *et al.*¹.

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Whistler mode wave packets in the Earth's foreshock region

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The definite identification of the basic modes of the variety of electromagnetic waves common in the interplanetary medium upstream of the Earth's bow shock has been difficult because of the Doppler shifting which results from their attempts to propagate in the supersonic solar wind. We have used magnetometer data from the dual ISEE-1 and -2 spacecraft, taken when the spacecraft were separated by a few hundred kilometres, to measure the apparent velocities of a subset of these waves. This has allowed the first determination of wave phase velocities, rest-frame frequencies, wavelengths, intrinsic polarizations and thus wave modes. We have now applied this technique to the class of upstream phenomena designated the discrete wave packets. These are isolated fairly monochromatic wave packets with frequencies ~ 0.4 Hz in the spacecraft frame which rotate in the left-handed sense about the magnetic field direction as observed in the spacecraft frame. Our studies of the time delay between the observation of such wave packets at the two spacecraft demonstrate conclusively that in the plasma rest frame these waves are, in fact, right-handed polarized waves with frequencies several times greater than the proton gyro-frequency, attempting to propagate upstream against the solar wind, but being carried towards the Earth by the solar wind flow.

The resolution of the ambiguity inherent in the study of waves propagating in a moving medium requires two point measurements. We have approached this problem by using data sets from the matching UCLA/ISEE magnetometers to attack this problem, using them to time the delay (Δt) between the appearance of a wavefront at first one, then the second, spacecraft. The wave velocity in the spacecraft frame ($v_{s/c}$) can then be found by dividing the distance travelled by the wave as it propagates between the spacecraft by the time taken to travel this distance. The distance itself is the projection of the spacecraft separation vector (*S*) in the direction of propagation (*k*), and the apparent velocity is thus

$$v_{s/c} = \frac{\mathbf{S} \cdot \hat{\mathbf{k}}}{\Delta t} \quad (1)$$

The separation vector is a well determined quantity (± 1 km) available from ISEE mission ephemeris tapes. The direction of wave propagation can be determined through eigenvalue analysis of the magnetic field variance matrix¹. The determination of Δt then allows $v_{s/c}$ to be calculated. The velocity is a combination of the intrinsic wave phase velocity (v_{ph}) and an additional component due to solar wind convection, which is equal to the projection of the solar wind velocity (*V_{sw}*) in the direction of wave propagation

$$v_{s/c} = v_{ph} + \mathbf{V}_{sw} \cdot \hat{\mathbf{k}} \quad (2)$$

Thus, once the solar wind velocity is known, the intrinsic wave phase velocity can be found by substituting the value of $v_{s/c}$ determined from equation (1) into equation (2) and solving for v_{ph} .

We applied this technique to a set of discrete wave packets observed by the ISEE-1 and -2 spacecraft on day 307 of 1977. The general characteristics of this variety of upstream wave were established by Russell *et al.*² in a comprehensive study based on single-spacecraft measurements, which we briefly summarize. The wave packets, which are most often found embedded in trains of irregular lower frequency (0.05 Hz) waves, typically grew rapidly in time and decayed more slowly, generally lasting two or three cycles. Wave packets were observed with frequencies throughout the interval chosen for study, 0.14–1.4 Hz with an average frequency of 0.4 Hz, and were left-hand polarized in 90% of the cases. From measurements of wave ellipticity, Russell *et al.* hypothesized that these waves were intrinsically right-hand polarized whistler mode waves of frequencies several times the proton gyrofrequency. Cold plasma theory predicts that such waves should have phase velocities of $\sim 100 \text{ km s}^{-1}$, which is significantly less than the typical solar wind velocity. Thus, if these waves were, as hypothesized, attempting to propagate upstream against the solar wind flow, they would be swept backwards over the spacecraft, resulting in the observed reversal of polarization. We present here direct experimental verification of this hypothesis.

The time period studied was 0200–0300 UT of day 307 of 1977 (Fig. 1). At this time, the spacecraft were in the interplanetary medium, inbound on the dawn side of the Earth at 1030 LT, several Earth radii (R_e) upstream of the final bow shock crossing which occurred at 0600 UT. The interplanetary magnetic field was essentially radial during this time period and the angle between the field and the nominal shock normal was 25° . Bursts of quasiparallel shock structure were encountered throughout the inbound portion of this orbit, and the studied interval is characterized by strong irregular magnetic field disturbances with time scales of 30 s, and by the presence of diffuse suprathermal ions (D. D. Sentman, personal communication). Magnetic field data used in this study were high-resolution measurements made by the dual UCLA magnetometers³ onboard ISEE-1 and -2. Data acquisition rate at this time was 4 vectors per s. Solar wind speeds were estimated using data from the University of Iowa's ISEE LEPDEA plasma analysers.

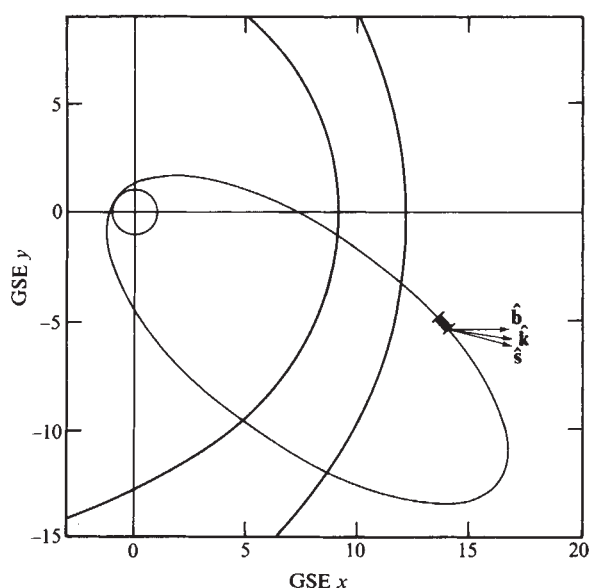


Fig. 1 Ecliptic plane projection of ISEE orbit with estimated shock and magnetopause positions on 3 November 1977. Location of satellites during the period of study, 0200–0300 UT, and ecliptic plane projection of the magnetic field, $\mathbf{b}$, the average wave normal, $\mathbf{k}$, and the model bow shock normal, $\mathbf{s}$, are also shown. The model for the bow shock used in this calculation is an ellipse with focus on the Earth and ellipticity 0.8.

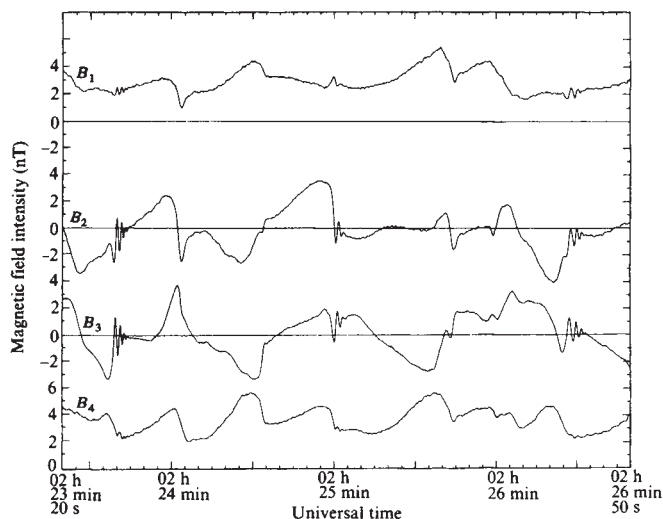


Fig. 2 The three vector components, in GSE coordinates, and the total magnitude of the magnetic field measured by the ISEE-2 magnetometer as the spacecraft was inbound on day 307 of 1977. The spacecraft was at a radial distance of $16.6 R_e$, upstream of the bow shock at 1030 LT.

Three typical wave packets are shown in Fig. 2. Note that the amplitude of these waves is a large fraction of the background field. As is the usual case, the packets (seen here beginning 02 h 23 min 39 s, 02 h 24 min 59 s and 02 h 26 min 25 s) are embedded in a train of lower-frequency waves and seen to be intimately related to the low-frequency wave structure, occurring when the field magnitude suddenly returns to low values after a more gradual increase. All the wave packets seen in the studied interval begin at the foot of sharp drops in the field magnitude (B_T), although not all such drops are accompanied by wave packets (for example, sharp decrease in B_T at 02 h 24 min 05 s). The field structure is thus apparently a necessary but not sufficient condition for the development of the packets on this day.

The profiles of the packet which begins at 02 h 23 min 39 s, as seen at the two separate spacecraft, are presented in higher resolution in Fig. 3. The spacecraft separation at this time was -117 , 180 and -107 km (GSE coordinates), with ISEE-2 located upstream and to the west of ISEE-1. The signals are nearly identical (the correlation is 0.92) except for a small but consistent time delay between the appearance of the wavefront at first ISEE-2 and then, 0.5 s later, downstream at ISEE-1. The precise time delay characteristic of the wave packets was determined from studies of the behaviour of the correlation between signals from the two spacecraft as a function of lag time, an example of which, for the wave packet shown in Fig. 3a, is shown in Fig. 3b. Correlation coefficients averaged over intervals appropriate to the signal duration were calculated for successive 0.25-s lags, consistent with the magnetometer's acquisition rate of 4 vectors per s. The correlation averaged over the 6-s duration of the wave packet peaks at 0.5 s, with secondary peaks appearing at 1.5-s intervals, consistent with the estimated wave frequency. The time delay expected for a static structure simply convecting with the solar wind flow would have been 0.3 s. The time delay can be determined to within $\sim \pm 0.1 \text{ s}$ using such plots, which at this spacecraft separation allows the resolution only of those wave phase velocities $\geq 70 \text{ km s}^{-1}$. Of the 24 wave packets observed in the hour 0200–0300 on day 307, 13 were characterized by velocities too small for us to resolve from the solar wind flow and the measurements on the remaining 11 cases are presented here.

Determination of the second element of the analysis, the distance travelled by the wavefront as it propagates between the spacecraft, requires determination of the direction of propagation of the wave. This direction can be determined within an

180° ambiguity by minimum variance analysis. Values computed by this technique independently from data from each of the spacecraft were in good agreement, and our analysis is based on averaged values. The time evolution of the field of the packet of Fig. 3a is shown in Fig. 4, where the variation is presented in the principal axes system. The field variation in the plane of the wavefront is plotted on the left. Note that the field rotates in the left-hand sense about the ambient field direction, which is into the paper in this case. As seen here, the wave

packets generally exhibit a nearly perfectly circular polarization. This same sense of rotation, left handed in the spacecraft frame, has been observed in all cases. The planarity of the wave is documented by the linearity of the right-hand plot, which shows the field variation in the plane formed by the direction of maximum variance and the wave normal. The nearly perfect plane circular polarization of this packet is typical and well justifies our identification of the direction of wave propagation with the minimum variance direction, which requires such

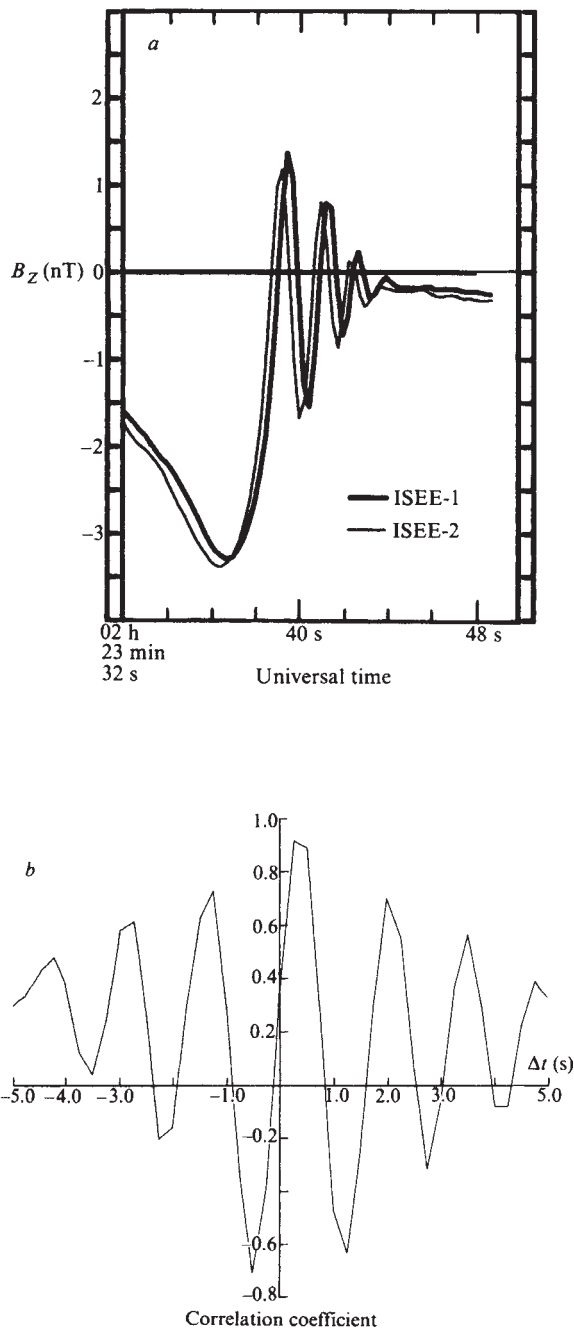


Fig. 3 *a*, Overlay of wave-packet signatures in the z component of the field of the two spacecraft for wave packet beginning at 02 h 23 min 39 s. *b*, The weighted correlation coefficient of signals from the two spacecraft as a function of imposed successive 0.25-s lag times (Δt). The correlation coefficient is the average lagged product of the times series on the two spacecraft for each of the three vector components weighted by the variance in that component. The correlation coefficients for each of the three components were quite similar. The correlation has been averaged over the 6-s duration of the 02 h 23 min 39 s wave packet. Positive Δt corresponds to signals which appeared first at ISEE-2.

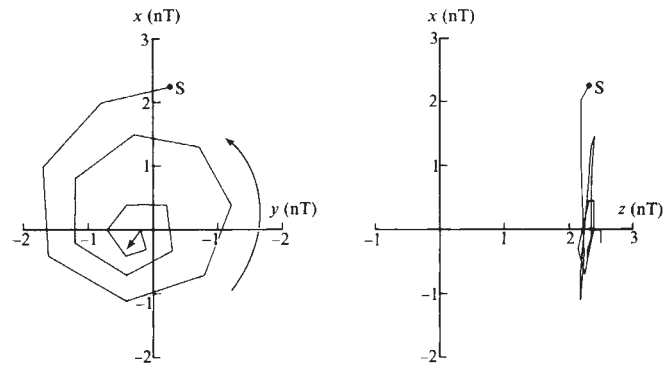


Fig. 4 The time evolution of the field vector of the 02 h 23 min 39 s packet, beginning at point S. The x , y and z axes correspond, respectively, to the directions of the maximum, intermediate and minimum variance eigenvectors.

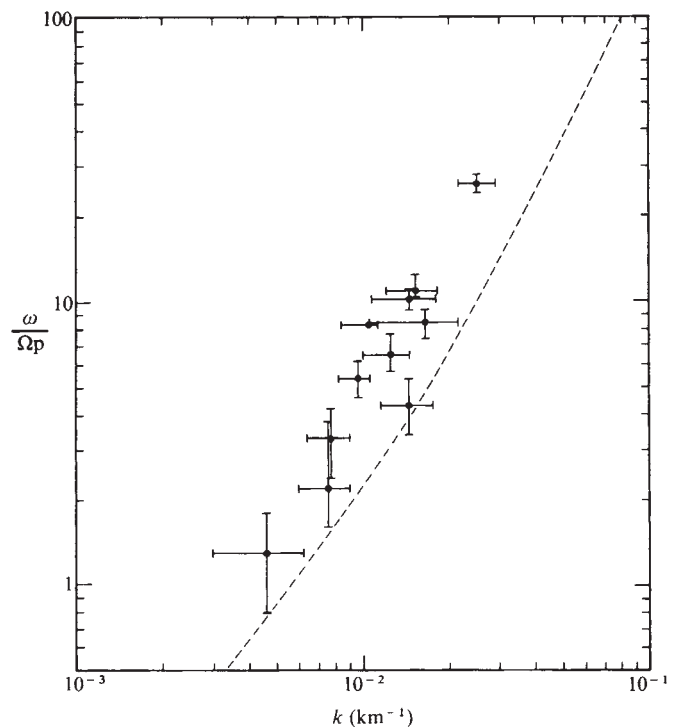


Fig. 5 Experimentally determined rest-frame frequencies, normalized to the local proton gyrofrequency, are plotted against wave number. Error bars represent uncertainties due to an estimated 0.1-s imprecision in Δt , which is the most significant source of error during this time interval. Also plotted (dashed line) is the relation expected from the cold plasma dispersion relation for a solar wind density of 3 cm⁻³, magnetic field magnitude of 3 nT and propagation angle of 25°, average values typical of this time period.

polarization for its validity. The direction of minimum variance is well determined in all cases. Typical ratios of the intermediate to minimum eigenvalue are ~ 50 , indicating that minimum variance directions are generally determined to within at least 1° . This direction tends to lie close to that of the ambient magnetic field with the typical angle between $\mathbf{k}$ and $\mathbf{B}$ being $\sim 25^\circ$.

From measurements of the wave frequency in the spacecraft frame (f_0) together with the wave velocity as determined above, one can also calculate the wavelength (λ): $\lambda = v_s/c/f_0$. The wavelength divided by the phase velocity then yields the plasma frame frequency (f_r): $f_r = \lambda/v_{ph}$. The results of such determinations for the wave packets studied here are presented in Fig. 5, where the angular frequency, ω , has been normalized to the local proton gyrofrequency, Ω_p , and plotted as a function of wave number k .

For comparison, we have presented the cold plasma dispersion relation for estimated ambient solar wind parameters and the 25° propagation angle typical of these packets, plotted as the dashed line in Fig. 5. The general trend of the data is quite consistent with the theoretical prediction although experimentally determined values tend to lie on the high side of the curve.

Theoretical approaches to these waves have been suggested previously by Wu⁴, Hasegawa⁵ and Gul'yel'mi and Bondarenko⁶ who, respectively, invoke wave-echo phenomena, a two-stream instability between the solar wind and magnetosheath plasma and MHD soliton formation. The extreme turbulence occurring both in the magnetosheath and in the foreshock region during quasispherical shock conditions as seen here argues against the feasibility of a plasma echo mechanism causing the waves. The present results are also inconsistent with both modes derived from the two-stream instability, which are a right-handed mode with $v_0 \gg V_{sw}$ and a left-handed mode propagating downstream. The agreement between the predictions of MHD soliton theory and wave packet characteristics found by Gul'yel'mi and Bondarenko is not confirmed by the present work.

In conclusion, the measurements present a consistent general picture of whistler mode plasma waves attempting to propagate upstream against the solar wind flow with phase velocities of the order of several times the Alfvén speed and wavelengths of the order of several hundreds of kilometres. Because the waves are being blown backwards over the spacecraft, their frequencies have been shifted from values several times the proton gyrofrequency in the rest frame to higher values in the spacecraft frame, and their intrinsic right-hand polarizations have become apparent left-hand polarizations at the spacecraft. The wave packets in this interval occur coincidentally with a sharp drop in the field strength which follows a more gradual rise in magnitude. As these structures are being blown downstream, the discrete wave packets are, in fact, on the upstream side of these lower-frequency structures, much like the whistler waves often seen at the foot of the bow shock^{7,8}. Thus, the low-frequency structures shown in Fig. 2 seem to be little shocks attempting to stand in the solar wind flow but being too weak and being blown backwards towards the Earth.

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Kinetics of void-lattice formation in metals

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Since the discovery¹ that voids form in neutron-irradiated stainless steel and that these could form into a three-dimensional lattice², void-lattices have been observed in some b.c.c. metals^{2,3} such as Mo, W, Nb, and Ta, and in f.c.c. metals^{4,5} such as Al and Ni. The irradiation conditions in which a void-lattice is formed are not understood⁶, but several explanations^{7–11} of the stability of the void-lattice have been given on the basis of void-void elastic interaction^{9–11}. Our present approach regards the void-lattice formation as a phase transition associated with the bifurcation of the homogeneous steady state in the mean field theory^{12,13}. The void-lattice is an interesting example of a class of phase transitions manifested by open, nonequilibrium, dissipative structures and have an inherent capability of self-organization. Although void-lattice formation has already been examined on this basis^{14,15}, we show here that the transition is induced in certain microstructural conditions where vacancy loop dynamics has an important role. The theory generally agrees with experimental features and predicts that another spatially dependent state exists which can influence irradiation-induced phenomena that have important technological implications for the development of fast reactor materials.

In the homogeneous rate theory^{12,13} the steady-state vacancy and interstitial concentrations C_v and C_i respectively, jointly represented by a column matrix C_p , are stable against any spatial deviations δC_p produced due to the fluctuations within the grain of size d . The stability¹⁶ is due to the presence of sinks like voids, vacancy loops, interstitial loops and network dislocations whose sink densities are represented by ρ_s , ρ_v , ρ_i and ρ_d respectively. If D_v and D_i are vacancy and interstitial diffusion constants, Z_1 the bias, $\beta_v = D_v(\rho_s + \rho_d + \rho_i + \rho_v)$, $\beta_i = D_i\rho_s + Z_1D_i(\rho_d + \rho_i + \rho_v)$ and α the recombination parameter, then the deviations δC_p in a linear approximation close to the steady state are governed by the equation

$$\delta C_p = (A_{11} + D\nabla^2)\delta C_p \quad (1)$$

where

$$A_{11} = \begin{pmatrix} -\beta_v - \alpha C_i & -\alpha C_v \\ -\alpha C_i & -\beta_i - \alpha C_v \end{pmatrix} \quad (2)$$

The matrix D is diagonal with elements D_v and D_i and the Laplace operator allows for point defect diffusion and the possibility of forming spatially ordered dissipative structures¹⁷. Using bifurcation analysis we choose a convenient orthogonal basis in terms of which the time and space evolution of any arbitrary deviation δC_p can be studied. We also examine how the basis functions themselves evolve and, therefore, attempt solutions of the type

$$\delta C_p = f_p e^{\omega_M t} \sin\left(\frac{m_1 \pi x}{d}\right) \sin\left(\frac{m_2 \pi y}{d}\right) \sin\left(\frac{m_3 \pi z}{d}\right) \quad (3)$$

where m_1, m_2, m_3 are integers $\neq 0$, f_p is a column matrix for the amplitudes and $\nabla^2 \delta C_p = M^2 \delta C_p$ with $M^2 = (\pi^2/d^2)(m_1^2 + m_2^2 + m_3^2)$. The boundary condition $\delta C_p = 0$ at the grain surface has been imposed consistent with the grain boundary being an effective sink. Further, after the bifurcation due to the spatial dependences, flows or currents $j_v = -D_v \nabla \delta C_v$ and $j_i = -D_i \nabla \delta C_i$ in the point defects will develop. These flows must be consistent with the symmetry of the host lattice. This amounts to imposing additional constraints on the indices m_1, m_2 and m_3 . If we now substitute equation (3) into (1) we obtain values of the frequencies ω_M which satisfy equation (1) and find that $\omega_M < 0$

for all values of sink densities ρ . This means that in this model the original homogeneous state is stable and any arbitrary deviations will decay to zero. In other words no soft mode $\omega_M = 0$ can arise because for no positive value of M^2 is the condition

$$\det |\mathbf{A}_{11} - \mathbf{DM}^2| = 0 \quad (4)$$

satisfied.

In the above analysis the response of the extended defect structure to changes in $\mathbf{C}_p$ has been ignored. This response is important and provides the additional interaction or coupling which leads to the bifurcation of the homogeneous state. This coupling arises mainly through the terms $\beta_V C_V$ and $\beta_I C_I$ which give the rate of loss of point defects to sinks. These terms are nonlinear when one includes the response of the microstructure as β_V and β_I are related through the sink densities to the sizes and concentrations of the extended defects. Let the column matrix $\delta \mathbf{C}_e$ represent the deviations δr_s in the void radius r_s and δN_v in the vacancy loop concentration N_v . These deviations will be functions of the spatial positions of the voids and vacancy loops. The contribution of interstitial loops can be included along with network dislocations under a suitable approximation. The equation of motion within the framework of the linear analysis is given by

$$\frac{d}{dt} \begin{pmatrix} \delta \mathbf{C}_p \\ \delta \mathbf{C}_e \end{pmatrix} = \left\{ \begin{pmatrix} \mathbf{A}_{11} & \mathbf{A}_{12} \\ \mathbf{A}_{21} & \mathbf{A}_{22} \end{pmatrix} + \begin{pmatrix} \mathbf{D}\nabla^2 & 0 \\ 0 & 0 \end{pmatrix} \right\} \begin{pmatrix} \delta \mathbf{C}_p \\ \delta \mathbf{C}_e \end{pmatrix} \quad (5)$$

where from the BEK theory¹³, $\mathbf{A}_{22}$ is diagonal with elements $\dot{r}_v/\bar{r}_v$ and $-\dot{r}_s/\bar{r}_s$; $\bar{r}_v$ is the initial radius of the vacancy loops. The coupling matrices $\mathbf{A}_{12}$ and $\mathbf{A}_{21}$ are functions of ρ_v and ρ_s . Following our previous arguments we again attempt solutions of the type given by equation (3) for $\delta \mathbf{C}_p$ and similar space dependent solutions for $\delta \mathbf{C}_e$ where the amplitude term $\mathbf{f}_p$ will now be replaced by a column matrix $\mathbf{f}_e$ for the extended defects. It is convenient, however, to partition the 4×4 matrix in equation (5) into two separate equations, one for $\delta \mathbf{C}_p$ and the other for $\delta \mathbf{C}_e$ using the standard partitioning techniques. Examining as before the possibility of a soft mode $\omega_M = 0$ we obtain now in place of equation (4) the condition

$$\det |\mathbf{A}_{11} - \mathbf{A}_{12} \mathbf{A}_{22}^{-1} \mathbf{A}_{21} - \mathbf{DM}^2| = 0 \quad (6)$$

This equation for certain values of ρ_v and ρ_s , which are the critical values for the transition, has solutions for positive values $M^2 = 3\pi^2/d^2$. This is the first bifurcation and represents the onset of the void-lattice formation.

Several experimentally observed features of the void-lattice formation emerge from this analysis. Figure 1 shows a phase

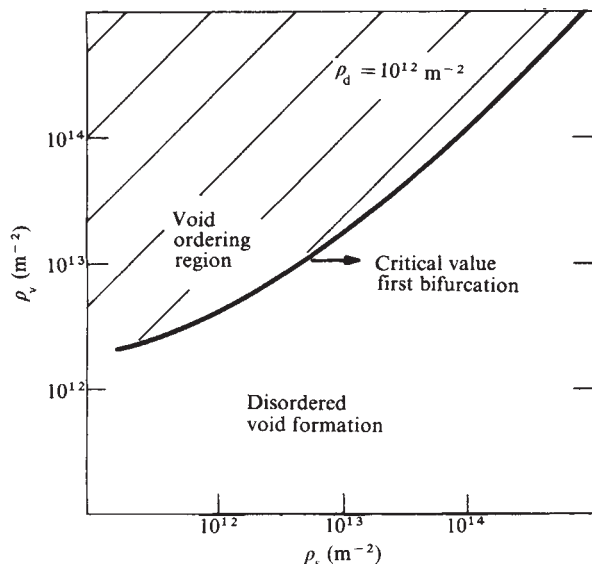


Fig. 1 Phase diagram showing where the first bifurcation appears. A vacancy migration energy of 1.3 eV has been used. For bifurcation and onset of void-lattice formation the system must evolve into the void ordering region.

diagram in the ρ_v, ρ_s space with the critical values of the densities obtained from equation (6) showing where the first bifurcation occurs. Whether the system reaches critical density values depends on how the system evolves in time and this has to be obtained by a solution of the BEK equations¹³. After the first bifurcation secondary bifurcations take place together with the ordering of the void-lattice. This introduces coarsening in the void distribution with correlations in void positions gradually emerging as is seen experimentally. The analysis of secondary bifurcations is difficult because of the stability factors coupled with the complex time and spatial dependence of the states involved. Such an analysis may have to be done by computer simulation techniques as in the case of simpler models which show similar self-organization behaviour¹⁷. Energy considerations as discussed by Malen and Bullough⁹ probably influence the final stages of void-lattice formation leading to the high stability of such a lattice. However, preliminary results show that as a function of ρ_v the parameter M^2 saturates at a value of about 10^{15} m^{-2} which, for a given grain diameter $d \sim 10^{-5} \text{ m}$, gives $m_1 = m_2 = m_3 = 100$, implying a void-lattice spacing of about 1,000 Å in reasonable agreement with experiments. The symmetry of the void-lattice must be the same as the host lattice because of the constraints imposed due to physical considerations on m_1, m_2 and m_3 arising due to the currents which develop after the bifurcation. However, a more fundamental approach, although difficult to analyse, would be to examine the factors which cause only those fluctuations which are consistent with the host lattice symmetry to grow. The phase transition involved is symmetry breaking from a homogeneous to a space periodic one. This explains why voids initially form randomly and only subsequently become ordered. The structure of the matrix $\mathbf{A}_{22}$ shows that $\dot{r}_v < 0$ is a requirement for bifurcation. The instability is therefore related to the vacancy loop formation. This is generally confirmed by experiments as electron-irradiation which produces voids effectively, but not vacancy loops, fails to give rise to regular void arrays. Such arrays are formed in both neutron and charged particle irradiation where vacancy loops are formed due to collapse of cascades. Note that no specific property of the vacancy loops is involved but rather the manner in which they couple with point defects. Such microstructural induced ordering effects can be expected in fairly general conditions in situations involving irradiation, stress or temperature gradients. It is in this context that the partial void ordering in nitrided steel¹⁸ by electron irradiation has to be viewed, where the possible trapping of vacancies by nitrogen gas atoms could produce the same effect as vacancy loops.

These results are interesting both because they explain many features of void-lattice formation, and because the possibility of a phase transition to a new spatially dependent state has important implications for several irradiation-induced phenomena which are of interest in the development of materials for fast reactors.

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Influence of modal composition on deformation in garnet lherzolite nodules

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A textural distinction has been drawn, in peridotites derived from kimberlite pipes, between relatively undeformed granular types and strongly deformed sheared types¹, that corresponds roughly with the absence and presence of large strained olivine grains (porphyroclasts) surrounded by smaller, recrystallized grains (neoblasts). More detailed classifications have also been proposed²⁻⁶. The textural variety is probably caused by both widespread mantle creep and more localized phenomena connected with kimberlite eruption⁷⁻¹². From consideration of dynamically recrystallized grain size¹¹ and dislocation densities¹², it seems that nodules that contain neoblasts have been deformed in high stress and strain rate conditions, over short periods of time, before eruption. The formation of the sheared textures and the eruption of the kimberlite are approximately simultaneous. I report here detailed textural observations on 10 garnet lherzolite nodules collected from kimberlite pipes in South Africa and Lesotho. They all have sheared textures, as defined above, showing a range from small amounts of grain boundary recrystallization to almost complete consumption of olivine porphyroclasts. Sections were cut parallel to the foliation and modal analyses were accurately determined by point counting one slide from each sample. The number of olivine porphyroclasts on each slide was then independently counted. The fewer the grains, the greater is the accuracy of estimating their number. Average porphyroclast areas were then calculated (Table 1). The data suggest that modal composition controls the extent of shearing.

Figure 1 shows the relationship between average porphyroclast area and percentage of olivine from the 10 thin sections. It seems that the lower the olivine content, the higher is the degree of deformation as recognized by the smaller areas of olivine porphyroclasts. The range of textures encompassed by the sheared group can be attributed to the range in olivine percentages.

This relationship can be justified by considering that the correlation will still hold if the sum of the other minerals present in the rock (pyroxene and garnet) is plotted along the x-axis. Kohlstedt and Van der Sande¹³ have estimated that the climb velocity of dislocations in enstatites is <0.1% of that in olivine. This behaviour results in a high creep resistance of orthopyroxene compared with olivine¹⁴ in the high temperature, hot working flow regime characteristic of these xenoliths. Despite recovery, strain rates are high enough to increase the strain energy at grain boundaries to a point where olivine begins to recrystallize while orthopyroxene continues to deform by slip

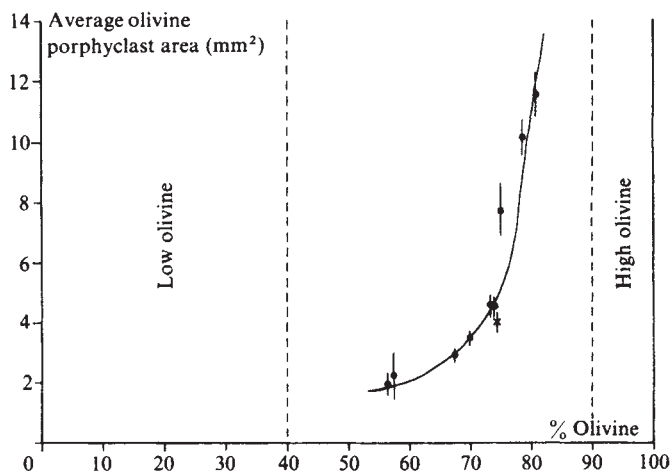


Fig. 1 Dependence of olivine porphyroclast grain size on amount of olivine after onset of recrystallization.

and rotation. This is confirmed in natural examples which show that enstatites, diopsides and garnets have a lesser tendency to polygonize and recrystallize²⁻⁵. Thus these minerals act as relatively rigid material while olivine recrystallization dominates. Where metasomatic phlogopite is seen to replace garnet on decompression it can also be included.

At the onset of deformation, stresses are maximized at porphyroclast grain boundaries as evidenced by recrystallization along them. If strain rates are high enough, concentrations of strain energies along these boundaries eventually cause recrystallization until the olivine porphyroclasts are entirely consumed. Olivine/olivine grain boundaries will be destroyed as neoblasts are produced along them while orthopyroxene/olivine, clinopyroxene/olivine and garnet/olivine boundaries will be relatively preserved. Thus strain energies high enough for olivine recrystallization will be retained at the latter boundaries between the rigid material and the olivine grains. Rigid/olivine boundary lengths will depend on modal percentages, hence percentage rigid material will determine available concentrations of strain energy, the release of which is accomplished by recrystallization.

The cross in Fig. 1 (nodule: BD2501) indicates a lower than expected area value. This sample has a higher percentage of garnet plus clinopyroxene, relative to orthopyroxene, than all the other samples, and optical evidence shows that these minerals are more resistant to recrystallization and disruption than orthopyroxene. Higher strain energies may have persisted at garnet/olivine and clinopyroxene/olivine boundaries while the pyroxenes began to recrystallize and so reduced the areas yet further. Similarly the square in Fig. 1 (nodule: XDB5) has no garnet and little clinopyroxene and has a high area value. The high olivine field (>90%) marks an area where recrystallization cannot continue after the onset of deformation. Indeed the

Table 1 Modal analyses and porphyroclast areas

Nodule	% olivine neophyroclast	% olivine porphyroclast	Slide area (mm ²)	No. olivine porphyroclasts	Porphyroclast area (mm ²)
BD2501	68.52 ± 0.5	5.88 ± 0.5	340	5	3.66–4.34
PHN2762	53.89 ± 1	3.41 ± 1	377	6	1.51–2.77
PHN2761	47.41	25.88	383	22 ± 2	4.13–4.96
KDT11	47.90	8.57	238	11 ± 2	1.57–2.27
KJAG22	0.80	79.85	444	31 ± 2	10.74–12.23
JJG859	54.85	15.05	700	30 ± 2	3.29–3.76
BD2344	48.08	19.23	480	32 ± 2	2.71–3.08
BD2384	15.86	62.59	566	35 ± 2	9.57–10.74
XDB5	0.53	74.68	440	43 ± 5	6.85–8.65
JJG703	2.00	71.71	427	68 ± 5	4.19–4.86

nodules showing the largest modal proportion of olivine (nodule: KJAG22) has only 0.8% neoblasts and shows olivine tablets of the type considered¹¹ to be formed by relief of strain energies during kimberlite eruption.

The existence of sheared dunites (>90% olivine) at Thaba Putsoa and the Frank Smith pipe⁸ and at Kampfersdam¹⁵ seems to conflict with the relationship in Fig. 1. The dunites found at Thaba Putsoa, however, are reported as having "porphyroclasts of olivine up to 1 cm in diameter" (very large). At the onset of deformation, and in suitable conditions, olivine/olivine grain boundaries will be destroyed and a certain percentage of recrystallized olivine will, hence, be present to exhibit porphyroclastic textures. The porphyroclast sizes will thus remain at a constant maximum to the right of the second dotted line in Fig. 1.

In contrast, deformation of dunites "to varying degrees"¹⁵ at Kampfersdam and other pipes cannot be explained by the above mechanism. The Kampfersdam dunites have been interpreted to be high temperature nodules due to the coarseness of their olivine groundmass. The recrystallization mechanism involving percentage of rigid material may only dominate within a certain range of conditions which are not, at present, possible to define, although current detailed geochemical studies (S. Beswetherick personal communication) may produce information. On the other hand, there may, in this case, have been a genuine externally imposed gradient in strain which is not evident in the specimens plotted on Fig. 1. The low olivine field (<40%) may indicate complete destruction of olivine porphyroclasts but the increased interaction of rigid/rigid grain boundaries as olivine percentage is reduced may tend to prevent further recrystallization.

An example of the modal compositional effects is shown by the single nodule (KDT11) which shows bands of high and low olivine contents. Figure 2 shows a grain map of this nodule in which the textural range of different samples is seen at the contact zone between bands. Larger olivine porphyroclasts tend to be restricted to the olivine rich bands while only neoblasts are seen in the areas where orthopyroxene predominates.

The above observations have important implications for the origin of the range of sheared textures we see at the surface. An externally imposed strain gradient is apparently not the only factor required to produce the spectrum of sheared textural types seen. The spectrum may be produced, at least partially, by compositional differences in the xenolith source regions. From phase considerations, production of basaltic liquids from garnet lherzolite results from partial melting in the approximate ratio, 1 olivine:5 orthopyroxene:10 clinopyroxene:10 garnet¹⁶. The

high orthopyroxene + clinopyroxene + garnet modal peridotites will correspond roughly with material less depleted in basalt, hence degree of partial melting may determine extent of shearing. Although granular nodules—those that do not contain recrystallized olivine—were not included in this study, note that in the least sheared samples studied the textures become similar to some granular types. The textural contrast between sheared and some granular forms may then be a transition related to extent of depletion. Before onset of deformation olivine grain sizes may have been considerably larger.

The present data indicate that the factor that controls extent of shearing, as recognized by consumption of olivine porphyroclasts by recrystallization, may be modal composition. From a structural point of view there is a genetic connection between degree of shearing and mechanical properties of the rock. It seems that the observed strain gradient would then be a function of internal compositional factors and not an external stress, temperature or strain rate gradient.

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A problem posed by vapour-dominated geothermal systems

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Vapour-dominated geothermal systems present an apparently extraordinary physical phenomenon—a layer of water lying stably on a body of steam. The first geothermal exploitation at Lardarello, Italy, was in such an area. We present here an analysis of the gravitational stability of water over steam in a porous medium. This shows that the near surface condensate layer of a vapour-dominated geothermal system can be stably maintained above the main steam reservoir by restoring forces associated with the displacement of the phase-change interface. For typical conditions in vapour-dominated geothermal systems, stability can be maintained provided that the permeability of the rocks at the depth of the steam-water boundary does not exceed about 40 nm² (0.04 millidarcy). The stability requirement determines the thickness of the condensate layer or the proximity of the top of the steam reservoir to the surface.

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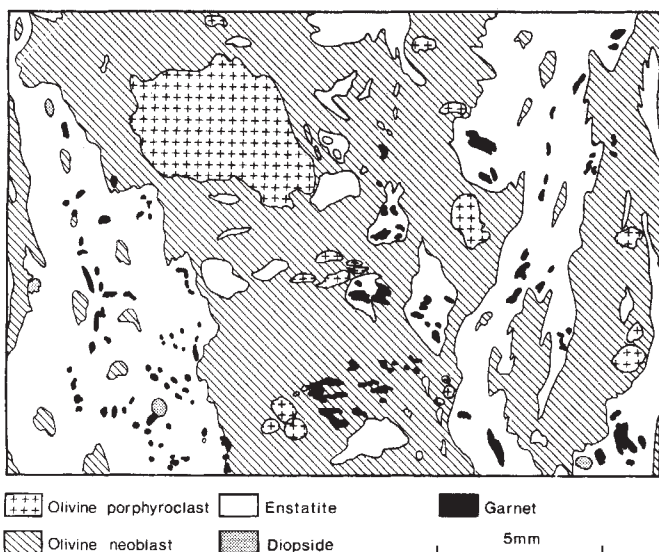


Fig. 2 Grain map of KDT11 showing compositional banding with preference of olivine porphyroclasts for olivine bands.

The basic structure of vapour-dominated systems is revealed by pressure measurements in wells drilled to different depths, as shown in Fig. 1 for the Kamojang geothermal field in Indonesia. The water table lies 100–150 m below ground surface. From there to 500 m depth, pressure increases as in normal ground-water, that is, approximately hydrostatically at a rate determined by the density of liquid water. Below 500 m depth, however, pressure increases only very slowly with depth; the rate of increase with depth is approximately that in a static column of steam. Pressure profiles similar to that in Fig. 1 have been published for the Lardarello geothermal field¹.

A section through the Kamojang field² (Fig. 2) illustrates the physical structure of a vapour-dominated geothermal system^{3–5}. A layer of brine, or very saline water, overlying a magmatic heat source, is presumed to exist at greater depth than shown in Fig. 2. The heat supply boils steam off from the brine, and this steam ascends through the fractured rock. It continues to rise until, at about 500 m depth, it meets the 'condensate layer', the liquid-dominated layer overlying the steam system. In the centre of the field some steam passes through this liquid, much like bubbles rising through water, and gives rise to a surface discharge of steam in fumaroles, steaming ground, acid springs and mud pools. Outside the central area of the field, steam does not escape from the steam reservoir.

Although drilling shows that steam is the pressure-controlling phase in the main reservoir of a vapour-dominated system, various observations demonstrate that the steam in this region must be in contact with some liquid water which is at or near residual saturation. The total production of steam at Lardarello is much greater than could be stored in a steam phase, although it could be accommodated as liquid⁶. At two of the vapour-dominated systems now explored, the geysers in California and Kamojang, although no longer at Lardarello, the fall in pressure caused by exploitation is accompanied by a fall in temperature, consistent with a temperature related to the steam–water saturation curve. Temperatures measured in the main steam reservoir of the Kamojang field (Fig. 1) agree with the saturation temperatures corresponding to the measured pressures. Measurements at the geysers also suggest that temperature increases with depth along the boiling point curve in the steam reservoir⁷.

In the liquid-dominated condensate layer, temperatures generally lie on a linear conduction profile^{2,7}. This is a consequence of both the surface temperature and the relatively low permeability of the near surface rocks as compared with

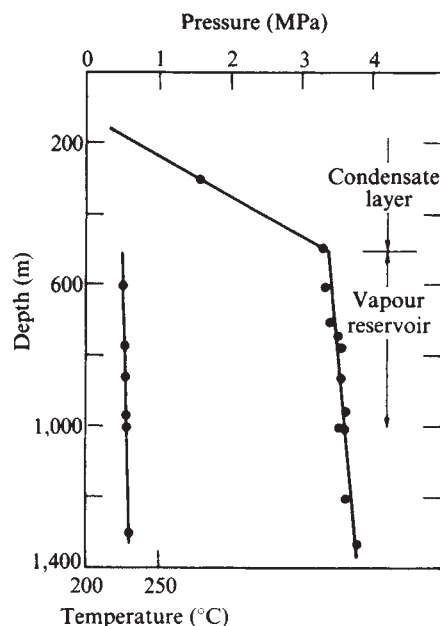


Fig. 1 Measurements of pressure and temperature in the Kamojang geothermal field in Indonesia (excluding the vicinity of well 7).

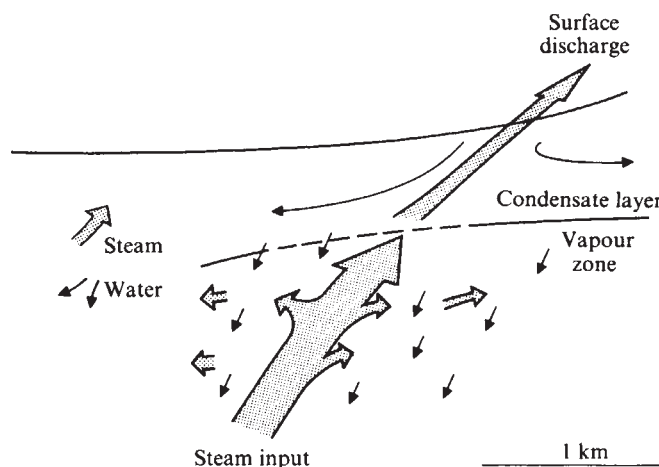


Fig. 2 Inferred structure of the Kamojang field².

those in the main steam reservoir. Where the near surface rocks are relatively more permeable, steam rises closer to the surface, and actually reaches it in some locations². In these areas, the temperature profile would lie on the saturation curve at all depths.

The major problem posed by the structure of Fig. 2 is why does the water not fall down? A sealed cap cannot separate the condensate layer from the steam reservoir because the natural discharge of the systems indicates that there is continuous permeability, at least in some places, between the mobile steam beneath and the mobile water above. If the water–steam interface behaved as a boundary between immiscible fluids, the configuration of water over steam would be gravitationally unstable for any permeability of the rocks in the vicinity of the interface, no matter how small. This can be understood by imagining a disturbance to the system which resulted in some downward flow of water towards the interface. If the water and steam were immiscible fluids, the boundary between them would be displaced downwards by such a disturbance. As a consequence, a vertical column of fluid in the region of the disturbance would contain more water and thus would be heavier than a column of fluid in the undisturbed quiescent state. The additional weight of the perturbed column provides a downward force tending to reinforce the original disturbance and displace the interface even further. Thus, the configuration is unstable.

A phase-change interface, however, distorts quite differently in response to such a disturbance because the interface must remain on the Clapeyron or equilibrium curve separating the phases. The location of a phase-change boundary is mainly dependent on temperature and pressure, and, unlike the case of the interface between immiscible fluids, flow can occur across a phase-change interface. Downward flow of water towards the steam–water boundary brings fluid to the interface that is colder than the fluid at the undisturbed location of the boundary as a consequence of the natural geothermal gradient. This causes the boundary to be displaced upward to lower pressure because it must remain on the Clapeyron curve. The phase-change interface distorts in a direction opposite to the sense of flow towards it, in striking contrast to the behaviour of the interface between immiscible fluids. As a result of the upward displacement of the phase-change boundary, a vertical column of fluid in the region of the disturbance would contain less water and thus would be lighter than a column of fluid in the undisturbed state. The reduced weight of the perturbed column provides an upward buoyancy force tending to oppose the original disturbance and stabilize the system. Another effect which tends to reinforce the stabilizing tendency of advection of colder water toward the phase change is that when water moves downward towards the phase change and the boundary moves upwards in response, there is a mass flow across the phase change which converts

water into steam. This phase transformation requires the absorption of the latent heat of vaporization and results in cooling of the surrounding fluid. As noted immediately above, cooling leads to upward displacement of the phase boundary and stabilization of the system. The cooling produced by the effects of advection and latent heat absorption promotes one destabilizing tendency associated with the thermal contraction of the water—colder water weighs more, and this additional weight of the column tends to drive the system in the same direction as the original downward perturbation.

To assess these effects quantitatively we have analysed the gravitational stability of horizontally infinite superposed fluid layers separated by a phase-change interface in a porous medium. The basic state consists of a motionless layer of water above a static layer of steam. Pressure increases with depth in the undisturbed state according to the hydrostatic law; the liquid and vapour densities govern the slopes of the pressure–depth profiles in the water and steam layers, respectively. Temperature is assumed to increase linearly with depth in the basic state. The stability of this configuration is governed by Darcy's law, which relates velocity and pressure perturbations, the incompressible continuity equation (density changes within each phase are negligible), which relates horizontal and vertical velocity perturbations, and the phase equilibrium equation, which relates pressure perturbations to the distortion of the water–steam interface. The differential equations for the disturbances are linearized and solved subject to the following conditions: (1) isothermal upper and lower boundaries, (2) no flow through the lower boundary, (3) constant pressure on the upper boundary, (4) continuity of pressure, temperature and mass flow at the water–steam interface, and (5) balance of conductive heat flux and latent heat release at the phase-change boundary.

The analysis shows that stabilization of water overlying steam requires

$$K < \frac{2k}{L \left(\frac{dp}{dT} \right)_c} \frac{\left(\frac{\bar{\mu}_l}{\bar{\rho}_l} + \frac{\bar{\mu}_v}{\bar{\rho}_v} \right) \left\{ \frac{\Gamma \left(\frac{dp}{dT} \right)_c}{g \bar{\rho}_l} - \frac{\left(\frac{\bar{\rho}_v}{\bar{\rho}_l} + \frac{\bar{\mu}_v \bar{\rho}_l}{\bar{\rho}_v \bar{\mu}_l} \right)}{\left(1 + \frac{\bar{\mu}_v \bar{\rho}_l}{\bar{\rho}_v \bar{\mu}_l} \right)} \right\}}{\left(1 - \frac{\bar{\rho}_v}{\bar{\rho}_l} \right)}, \quad (1)$$

where ρ is density, μ is dynamic viscosity, $(dp/dT)_c$ is the slope of the Clapeyron curve, L is the latent heat, Γ is the geothermal gradient, g is the acceleration of gravity, k is the combined thermal conductivity of the fluid and matrix (dominated by the conductivity of the rocks), K is the permeability, and subscripts l and v refer to liquid and vapour, respectively. All the quantities in equation (1) are to be evaluated at the quiescent position of the water–steam interface. Condition (1) is necessary for stability. Although it is not sufficient, an exact analysis⁸ shows that the value of permeability given by the right side of equation (1) is a good estimate of the maximum permeability for which stabilization is possible.

The evaluation of equation (1) leaves little uncertainty in the largest value of permeability allowing stabilization of the condensate layer. This is because all the quantities in equation (1), except for k and Γ , depend only on temperature, and there is little temperature variation within and among the steam reservoirs of natural vapour-dominated systems. In addition, the condensate layers are generally several hundred metres thick, precluding any large uncertainty in the value of Γ . Finally, the thermal conductivities of reservoir rocks are known to within factors of 2 or 3. Using a value of 242 °C for the temperature at the condensate layer–steam zone interface (see Fig. 1), $\Gamma = 0.555 \text{ °C m}^{-1}$ and $k = 4 \text{ J m}^{-1} \text{ s}^{-1} \text{ K}^{-1}$, we find that K must be less than 80 nm² (0.08 millidarcy) for the condensate layer to be gravitationally stable [a more exact analysis⁸ shows that $K \leq 40 \text{ nm}^2$ (0.04 millidarcy) for stability].

A permeability of 40 nm² is more than an order of magnitude smaller than permeabilities generally thought to occur in the main reservoirs of geothermal systems. It is not surprising, however, that relatively low permeability is required near the

condensate layer–steam zone interface to keep the water from falling down into the underlying region of steam. The significance of our result is the fact that any stabilization is possible for a non-zero value of permeability. The natural discharge of vapour-dominated geothermal fields attests to the through-flow of steam within the system. This could not occur if some impermeable layer were required to seal or cap the main steam reservoir to stabilize the condensate layer. It is the special behaviour of the phase-change interface between water and steam that permits stabilization of the systems with a non-zero permeability; if the interface behaved in a manner similar to the boundary between immiscible fluids the rocks would have to be impermeable to keep the condensate layer from falling down.

The low permeability required for the stability of the condensate layer probably determines the thickness of the layer. During the formation of a wet geothermal system, rising steam from the relatively permeable main reservoir penetrates rocks whose permeability decrease with proximity to the surface. The top of the main reservoir will extend upwards until condensate can be stabilized by rocks of sufficiently small permeability.

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The Gulf of Lion: subsidence of a young continental margin

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The origin of the subsidence of Atlantic-type continental margins is of much interest to geology and geophysics. The main problem at these margins is in explaining the substantial thicknesses of mainly shallow-water sediments which accumulate soon after rifting and plate separation. We have now used biostratigraphic data from commercial wells to study the subsidence history of a young Atlantic-type continental margin. Backstripping the sediment load reveals that the margin has been subsiding at nearly mid-ocean ridge rates. Geological data indicate that lithospheric stretching alone cannot account for this amount of subsidence and other processes must be involved.

Studies of biostratigraphic data from deep commercial boreholes suggest that the tectonic subsidence of margins is thermal in origin^{1–3}. Sleep¹ suggested the subsidence is due to cooling of the lithosphere following uplift and erosion. This model cannot, however, fully explain the amount of tectonic subsidence observed at these margins³ or the lack of evidence of domal uplift during rifting⁶. McKenzie⁷ proposed a model in which the subsidence is due to cooling of the lithosphere following stretching and thinning at the time of rifting. This model seems to explain the tilted and rotated blocks bounded by listric faults which develop during rifting^{8,9}.

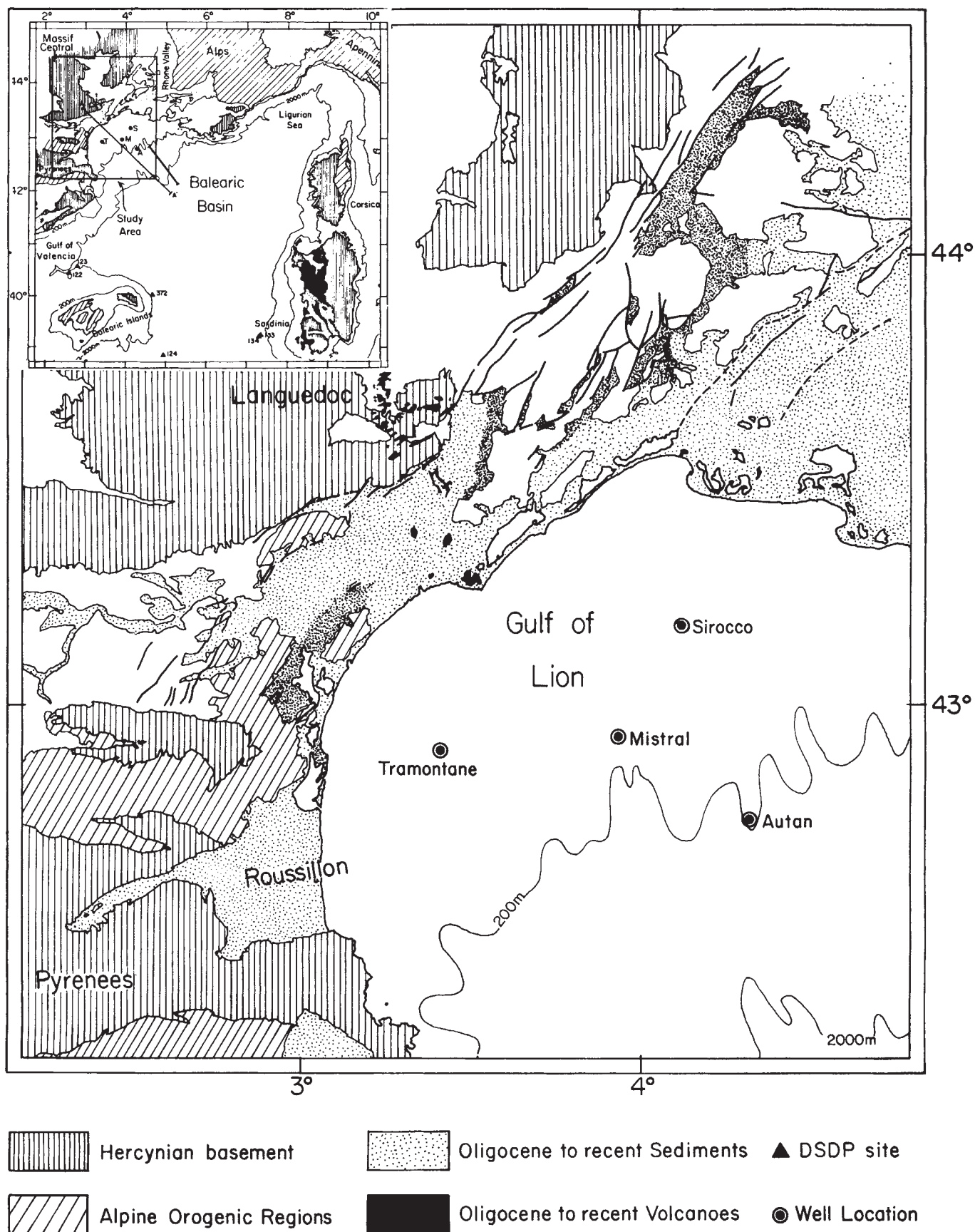


Fig. 1 Geology of the Gulf of Lion. The heavy lines are faults which have been active during the Oligocene, dashed where inferred. The heavier dots show the location of Oligocene red beds. Inset, generalized geology of the regions surrounding the Balearic Basin and location of Gulf of Lion. The heavy line is the location of the 'Christiane' multichannel seismic profile^{30,32}, A-A' is the location of the profile shown in Fig. 2.

By quantitatively accounting for the effects of sediment and water loading the tectonic subsidence of a margin can be isolated^{2,4}. To correct for sediment and water loading, the sedimentary section should be reconstructed at intervals during the development of the margin, taking into account the effects of compaction, changes in water depths and eustatic sea-level changes. The sedimentary section should then be isostatically unloaded, or backstripped^{2,3}.

Most previous studies have been based on stratigraphic data from the thickly sedimented, relatively old margins, such as eastern North America. The wells in these studies generally did not sample the pre-rift and syn-rift sediments or crystalline basement rocks and therefore do not provide either a complete record of the subsidence history or a reliable age for the opening of the central North Atlantic. A detailed knowledge of the early subsidence history is important in discriminating between different models for the origin of the margin.

A good example of a well sedimented young Atlantic-type continental margin is the Gulf of Lion in the Balearic Basin (Fig. 1). Geophysical data indicate that the Balearic Basin contains up to 6 km of sediments, including over 1 km of Messinian evaporites, overlying a thin oceanic-type crust¹⁰⁻¹². Geological and palaeomagnetic evidence^{11,13-16} suggests the basin formed by Oligocene/Miocene rifting of Corsica-Sardinia from France (Fig. 1). Messinian evaporites had been interpreted¹⁷ as indicating that the western Mediterranean was not a deep basin until post-Miocene time. However, DSDP site 372 east of Menorca¹⁸ established that the western Mediterranean was already a deep basin by the Lower Burdigalian (~20 Myr BP). Although the tectonic setting of the basin is poorly known, it may have formed as a back-arc basin behind a migrating island arc-trench system to the east¹⁶. Extensive Oligocene through Lower Miocene andesitic volcanism related to this arc is found in western Sardinia¹⁹.

Figure 1 summarizes the geological setting of the Gulf of Lion. The Eocene in southern France was a period of overthrusting associated with the Pyrenean orogeny^{20,21}. The Oligocene, in contrast, was a period of extension and normal faulting^{20,21}, much of which seems to have reactivated older fault trends.

Continental sediments accumulated in NE-SW trending grabens in Languedoc and Roussillon. Similar extension and graben structures also occurred in Sardinia and along the margins of the Balearic Islands^{22,23}. Drainage patterns in southern France during the Eocene and Oligocene were towards the north and west, away from the present Mediterranean^{24,25}. Then, in the Lower Miocene (Aquitainian) a major transgression occurred marking the opening of the Balearic Basin^{26,27}.

The structure and stratigraphy of the Gulf of Lion margin is known from commercial drilling and seismic reflection data. Three of the wells drilled recovered over 3 km of Miocene to Recent sediments overlying Palaeozoic basement^{28,29}. In addition, the Autan well (Fig. 1) recovered 244 m of ?Oligocene red beds²⁹. The seismic data in the Gulf of Lion³⁰⁻³³ show a gently dipping Pliocene to Recent sedimentary section unconformably overlying a more steeply dipping Miocene section. Information on the basement structure of the Gulf of Lion is sparse but available data^{28,33} indicate the Miocene to Recent sedimentary section overlies horst and graben structures which are infilled by continental sediments of probable Oligocene age (Fig. 2). Thus, the available geological and geophysical data show that the structural evolution of the Gulf of Lion region margin was similar to that of a normal Atlantic-type continental margin¹².

We have used biostratigraphic data from three wells²⁹ in the Gulf of Lion (Figs 1, 2). The sediments in each well were backstripped^{2,3} to obtain the tectonic subsidence of the margin. The wells were corrected for compaction using available sonic, density and porosity logs (L. Montadert, personal communication) and backstripped with the Airy model of isostasy^{2,34} and a mantle density of 3.33 g cm^{-3} . The palaeobathymetry for the wells was based on the micropalaeontological studies of Cravatte *et al.*²⁹. We did not correct for eustatic sea-level changes as they are not well known in the Neogene due to uncertainties in the timing of Antarctic glaciation.

The sedimentary record in the wells is interrupted by a major erosional unconformity due to the Messinian salinity crisis^{30,35} (Fig. 3). The unconformity is observed as a discontinuity in the lithological logs. We backstripped each well using as wide a range of estimates for the magnitude of the erosional event as

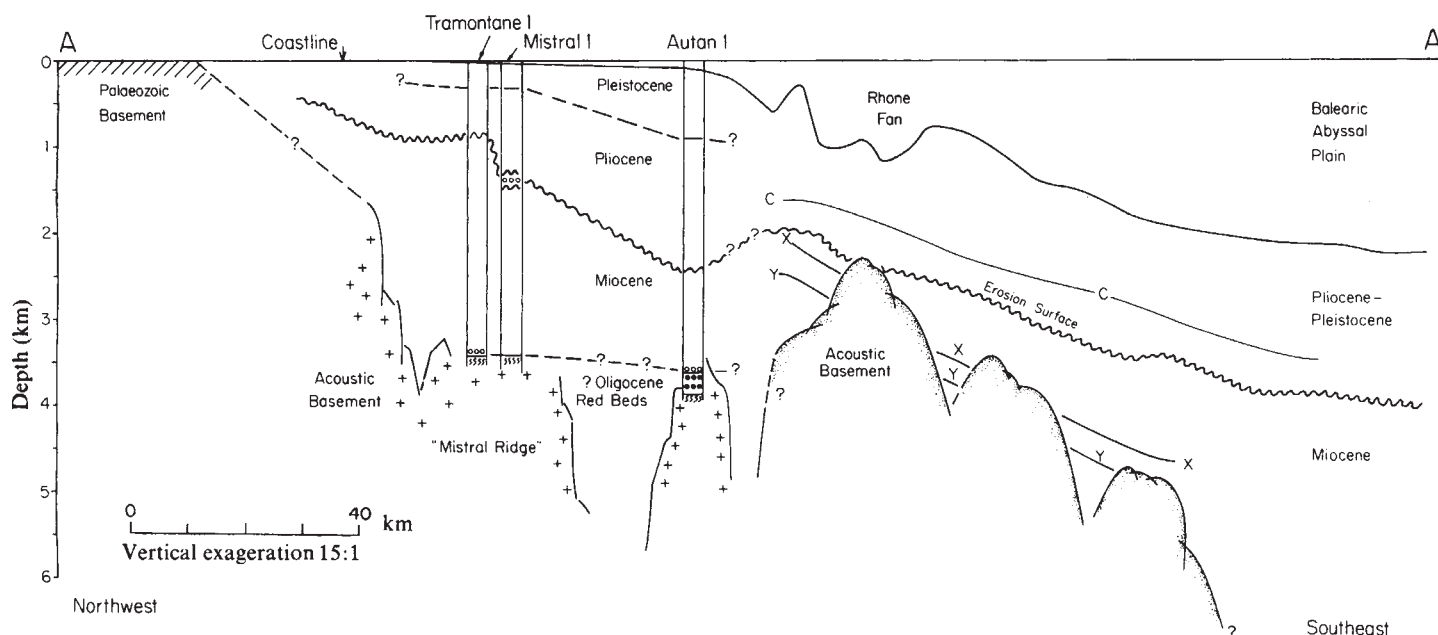


Fig. 2 Profile across the Gulf of Lion margin. Prominent seismic reflectors and acoustic basement are projected from the 'Christiane' profile³⁰. In the wells: ○, basal conglomerates; ●, continental red beds. The wells bottomed in Palaeozoic schists^{28,29}. The basement structure suggests prominent horsts and grabens infilled by red beds (refs 28, 33 and L. Montadert, personal communication) seaward of a hinge zone.

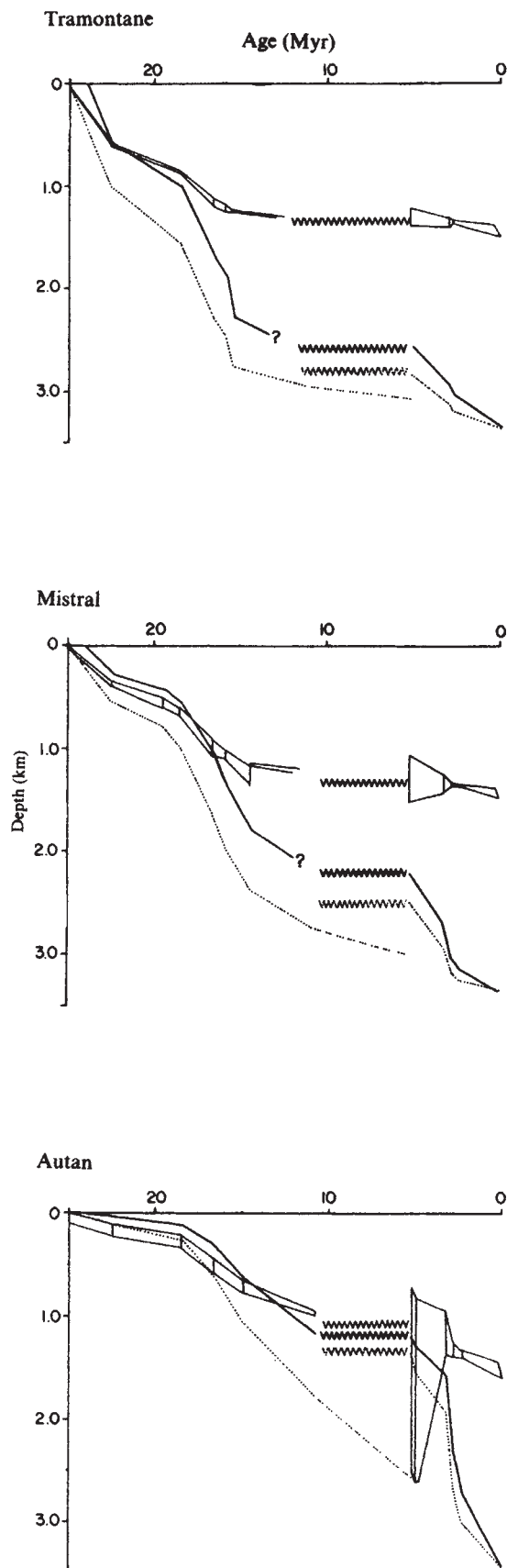


Fig. 3 Subsidence in the three wells studied. The heavy solid lines are the observed sediment thicknesses. The dotted lines represent the reconstructed sediment accumulation after correction for compaction, dashed where later removed by erosion. The double solid line is the subsidence after backstripping. The width of the lines represent the range of estimates for the water depth based on micropalaeontological studies²⁹.

was compatible with the well logs and found that in each case the trend of the tectonic subsidence (Figs 3, 4) was continuous through the salinity crisis. This agrees with the interpretation of the Messinian event as an evaporitic drawdown^{32,35}.

Using the observation that the tectonic subsidence was continuous, it is possible to estimate the amount of erosion of the margin during the salinity crisis. We found that 1,200–1,500 m of sediments were eroded at Autan and that much less were eroded at the more landward Mistral (380–600 m) and Tramontane (<320 m) wells. The water depths immediately following the salinity crisis were estimated to be 700–900 m at Autan and correspondingly smaller at the more landward wells, in agreement with those determined from micropalaeontological studies²⁹. At the Autan well, however, by using the trend of the tectonic subsidence we can estimate a much smaller range of water depths than that which can be estimated from micropalaeontology (bathyl).

The tectonic subsidence at each well (Fig. 3) constitutes less than half of the total sediment accumulation and continues smoothly through the Messinian event. The tectonic subsidence is similar in form for each of the wells. The main difference occurs in the early subsidence which is larger in a landward direction. Small variations in the rate of the tectonic subsidence appear to correlate from well to well and may be due either to changes in sea-level³⁶ or to uncertainties in absolute age dating of the sediments.

The most likely cause of the tectonic subsidence is thermal contraction following stretching and thinning of the margin at the time of rifting. A useful way to examine the thermal subsidence of a margin is to plot the tectonic subsidence against $\sqrt{\text{age}}$ (ref. 7). Figure 4 shows the tectonic subsidence for the wells along with that predicted by a lithospheric stretching model⁷. The origin in Fig. 4 is 25 Myr BP which is the best estimate for the age of opening of the basin^{13,14,16,27}. The parameter $\beta = \infty$ is equivalent to the subsidence of a mid-ocean ridge.

The total tectonic subsidence at each well is large and seems to exceed $\beta = 10$. The fit of the subsidence data to the model, however, is poor. The tectonic subsidence early in margin history is progressively greater in a more landward direction and at Tramontane exceeds that of a mid-ocean ridge.

Numerical modelling of the continental margin off Norway³⁷ has shown that lateral transport of heat towards the continent may be important in the early history of the margin. We have, therefore, modified McKenzie's⁷ model to include a finite crustal thickness for oceanic lithosphere and the effects of lateral as well as vertical conduction of heat. The subsidence was computed for a two-dimensional model in which β varied across the margin (Fig. 4b). The fit to the subsidence data has been greatly improved (Fig. 4a) and the estimates of β (2.6 to 6) at the wells reduced. These considerations suggest that thermal cooling, both vertical and lateral, of a hot, thinned lithosphere can explain the tectonic subsidence of the margin.

The magnitude of the tectonic subsidence implies that the continental basement in the Gulf of Lion was greatly extended during rifting. Extension of this magnitude, however, does not seem to be compatible with the geology of the Gulf of Lion. Normal faulting and graben structures containing Oligocene red beds occur both on land^{20,21} and off-shore (ref. 33 and L. Montadert, personal communication). However, the volume of these pre-rift and syn-rift sediments is relatively small compared with the volume of the post-rift sediments (Figs 1, 2). All the wells drilled in the Gulf of Lion (Fig. 1) contain little or no Oligocene sediments (Fig. 2) and are located on horsts. If the lithosphere had been stretched by the estimated values of β then the sediment accumulation during rifting should have been in excess of 4 km. Thus, the small observed subsidence associated with rifting precludes large amounts of stretching of continental crust, while the magnitude of the thermal subsidence requires extensive heating of the lithosphere during rifting.

These results suggest that although there was extension during the Oligocene, the passive heating resulting from stretching⁷ cannot fully account for the observed tectonic subsidence of the

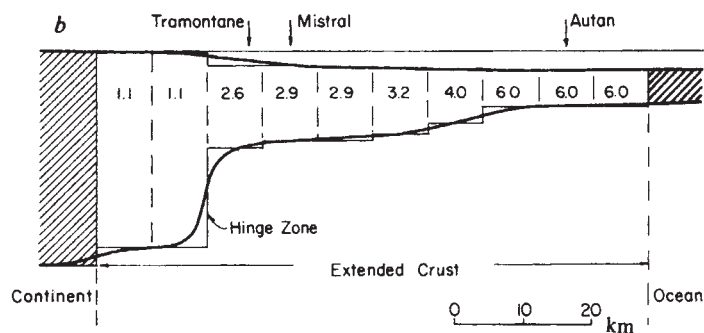
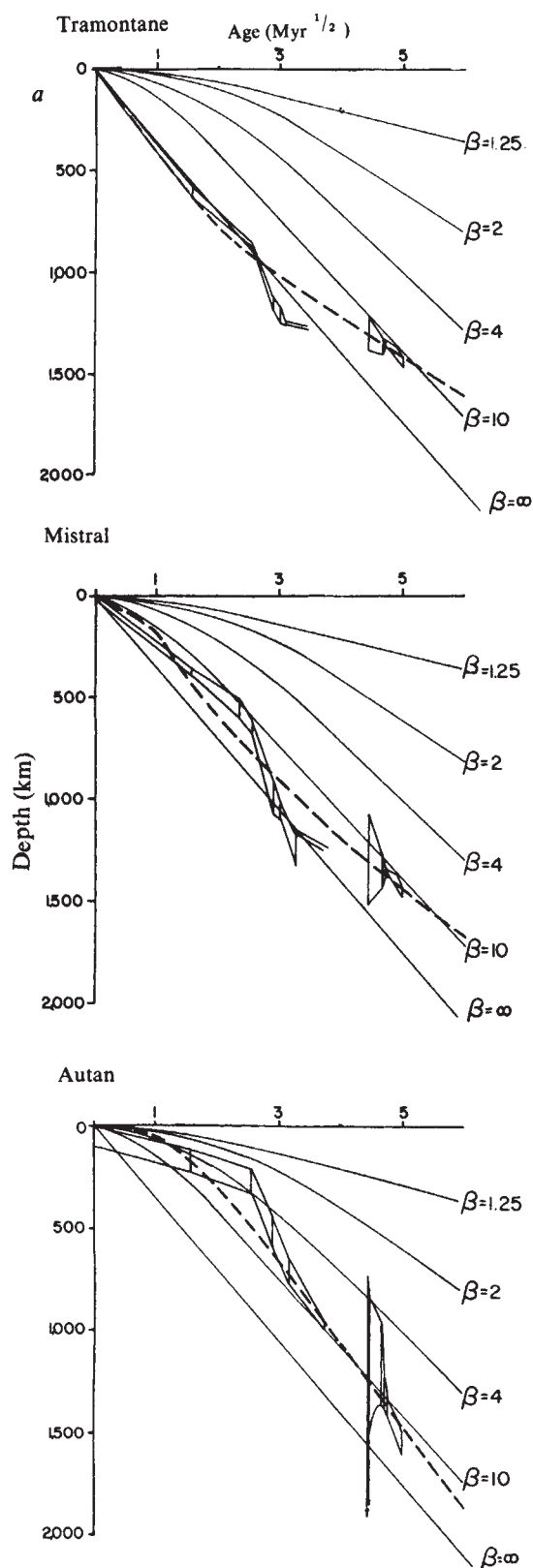


Fig. 4 *a*, Tectonic subsidence plotted against square root of the age. Width of lines represents range of estimates for water depth²⁹. Age of opening was assumed to be at 25 Myr BP. Light lines, theoretical subsidence calculations from lithospheric stretching model⁷. $\beta = \infty$ corresponds to mid-ocean ridge subsidence. Dashed lines, subsidence calculations for two-dimensional model illustrated in *b*. Crustal section of the two-dimensional model used to estimate subsidence at the wells. Dashed lines represent boundaries of the blocks used for calculations. The values for each block are the estimates of β . The heavy lines illustrate the crustal thinning that occurred at the margin at the time of stretching (25 Myr BP). Note the large values of initial water depths. No vertical exaggeration.

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margin. Thus, other mechanisms of heating the margin at the time of rifting are required. One possibility is that a thick crust and relatively high thermal gradients already existed in the lithosphere before rifting, due to Pyrenean Eocene overthrusting and/or andesitic volcanism in the nearby island arc-trench system. Alternatively, continental rifting may be caused by deep seated processes in the mantle which result in an active heating of the lithosphere at the margin. Further work on other Atlantic-type continental margins is needed to distinguish between these possibilities.

Model experiments on the 100,000-yr glacial cycle

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It is believed that during the Quaternary era changes in global ice volume were mainly due to changes in the size of the ice sheets on the Eurasian and American continents. Time spectra of oxygen isotope records from deep-sea cores and of the Earth's orbital parameters are remarkably similar in the 10,000–120,000-yr range^{1,2}, suggesting that changes in global ice volume are forced by insolation variations. Model studies by Weertman^{3,4} and Pollard⁵ have confirmed this point to some extent: the 20,000- and 40,000-yr cycles can be reproduced, but the 100,000-yr cycle does not show up. Recently, Imbrie and Imbrie⁶ have fitted simple nonlinear mathematical models to $\delta^{18}\text{O}$ curves. They found that reasonable fits are obtained if the time scale for ice-sheet growth is about 27,000 yr and for decay about 7,000 yr. The present study considers the problem of the 100,000-yr cycle in a similar way. Experiments with a Northern Hemisphere ice-sheet model show that the 100,000-yr cycle and its sawtooth shape may be explained by ice sheet/bedrock dynamics alone. This cycle seems to be an internally generated feature and is not forced by variations in the eccentricity of the Earth's orbit.

First, consider the 'classical' record of core V12-122 (Fig. 1). This core has a comparatively high resolution and spans about the last 500,000 yr. Two features that are immediately evident are that the dominant signal is a periodic one, with a period of about 100,000 yr, and that this cycle is strongly asymmetric, having a sawtooth shape. The strong response of the cryosphere on the 100,000-yr time scale, as compared with those on the 20,000- and 40,000-yr time scales, suggests some kind of resonance. Variations in eccentricity of the Earth's orbit may have combined with the internal dynamics of the climate system to create a strong signal in global ice volume.

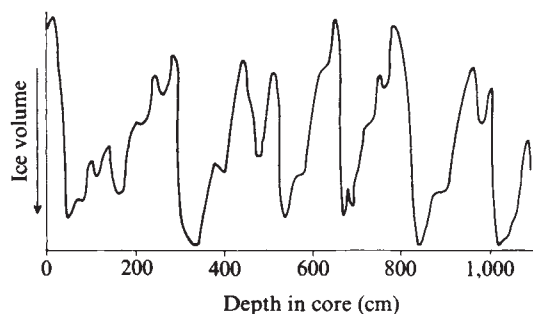


Fig. 1 A plot of the $\delta^{18}\text{O}$ record of the Carribean core V12-122 (data from ref. 12). According to the 'tune-up' time scale of Hays *et al.*², the glacial maximum found at a depth of 840 cm in this core occurred at 430,000 yr BP. The most pronounced features are the 100,000-yr cycle and the asymmetric shape of these cycles.

Figure 1 suggests that the time needed to create full-size ice sheets is 50,000–100,000 yr, considerably more than has been suggested by theoretical studies^{4,7}. I will discuss some model results indicating that such a growth time scale is in accordance with bedrock/ice sheet dynamics.

The most dramatic feature of Fig. 1 is the speed with which deglaciations seem to take place ~10,000 yr. Also important is

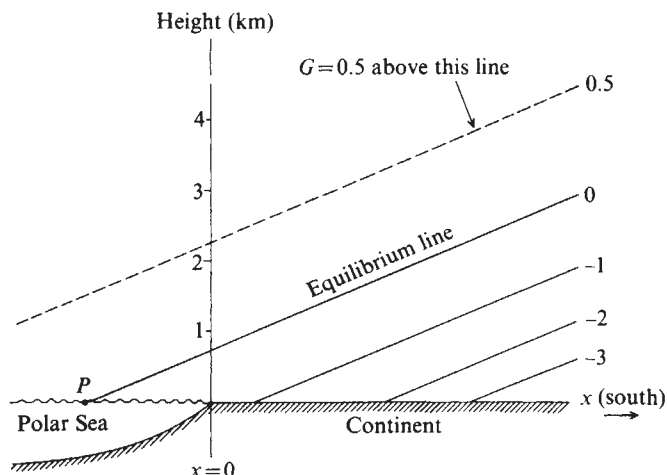


Fig. 2 Mass-balance conditions for a Northern Hemisphere ice sheet, as parameterized in the model. The intersection of the equilibrium line with sea level is called the 'climate point', and is indicated by *P*. Insolation variations are represented by shifting *P* north- and southwards. Values of the constants used are: $a = 0.73 \times 10^{-3} \text{ yr}^{-1}$, $b = 0.27 \times 10^{-6} \text{ m}^{-1} \text{ yr}^{-1}$, $\chi = 0.65 \times 10^{-3}$. Values for *G* are given beside each line.

the fact that just before deglaciation starts, the growth rate of the ice volume does not fall, which indicates that during the glacial maxima the Northern Hemisphere ice sheets were far from equilibrium. Also, the difference in $\delta^{18}\text{O}$ between a glacial minimum and the subsequent maximum is fairly constant, suggesting an inherent instability of the climate system, and probably of the cryosphere.

A mechanism that might explain this instability is bedrock sinking. Weertman⁴ first suggested this as a possible cause for rapid deglaciation in connection with the Northern Hemisphere ice sheets. I will now discuss model experiments which deal with this point.

The Northern Hemisphere ice-sheet model to be used is described by the equations⁸

$$\frac{\partial H}{\partial t} = \frac{\partial}{\partial x} \left[D \frac{\partial (H+h)}{\partial x} \right] + DH/Y^2 + G \quad (1)$$

$$\frac{\partial h}{\partial t} = -\alpha(H+3h) \quad (2)$$

$$G = a(H+h-E) + b(H+h-E)^2 \quad (3)$$

Equation (1) formulates the conservation of ice mass; it is essentially a nonlinear diffusion equation. The diffusivity for ice mass *D* increases monotonically with ice thickness *H* and the slope of the ice surface $\partial(H+h)/\partial x$ (where *h* is the bedrock height with respect to some geopotential level). The *x*-axis points in a southward direction, so the evolution of the ice sheet is explicitly computed along a meridian. The term DH/Y^2 governs lateral ice-mass discharge, where *Y* is a characteristic east-west length scale of the ice sheet. *G* is the mass balance of the sheet, and is a function of height with respect to the equilibrium line [see equation (3)]. The height of the equilibrium line (defined by *G* = 0) is denoted by *E*; it increases linearly with *x* at a rate χ (Fig. 2).

Equation (2) deals crudely with isostatic adjustment of the Earth's crust. In the equilibrium case, $h = -H/3$ corresponds to a rock density of three times that of ice. The time scale of adjustment is given by $1/3\alpha$.

Figure 2 shows in detail the appearance of the mass balance. Insolation variations may be interpreted as shifting the equilibrium line northwards and southwards. During periods with large eccentricity of the Earth's orbit, the amplitude of the 'climate point' *P*, due to precession of the equinoxes, is about 10° in terms of equivalent latitude⁹. Only during such periods does *P* reach the continent; normally, it is located in the Polar Sea.

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If P lies on the continent for some time, an ice sheet will start to grow. Due to the feedback between surface elevation and mass balance the ice sheet may continue to grow even if insolation increases and P returns to the Polar Sea. Bedrock sinking, however, attempts to cancel this feedback.

I have carried out several numerical integrations of equations (1)–(3). The model was solved on a grid with 70 km spacing, the lateral scale Y being 1,000 km. (For more technical detail, see ref. 8.) In the first experiment, a periodic forcing was imposed by varying the position of the climate point according to

$$P = -140 + 490 \times \sin(2\pi t/T) \text{ km} \quad (4)$$

Remember that $P < 0$ implies that P is located in the Polar Sea. T was set to 20,000 yr, so equation (4) roughly represents conditions during maximum eccentricity. The precise form of the forcing function seems to be relatively unimportant—once the ice sheet reaches a height of ~ 500 m, ice-sheet dynamics take over. Thus, the only requirement is that P remains for a sufficient time on the continent. In view of this, our model can be kept simple by using equation (4) as a universal forcing function in this experiment.

Figure 3 shows four model runs with various time scales for isostatic adjustment (T^*). Figure 3a ($T^* = \infty$) shows the case without any movement of the bedrock. The ice sheet grows slowly, then more rapidly, and comes into a stable oscillation with quite small amplitude. A huge increase in the height of the equilibrium line would be required to remove the ice sheet.

Figure 3b and c show what happens if adjustment takes place on time scales of 30,000 and 10,000 yr, respectively. The ice sheet grows at a smaller rate now, and after about 100,000 yr disappears 'spontaneously'. Apparently, the sinking of the

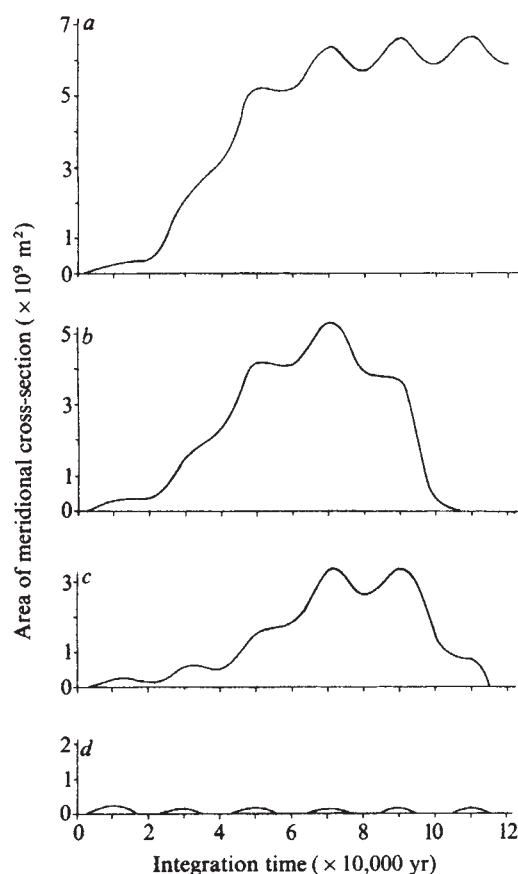


Fig. 3 Model runs with various time scales of isostatic adjustment (T^*). The area of the meridional cross-section through the ice sheet is given along the vertical axis. To obtain the ice-sheet volume, this should be multiplied by a characteristic east-west extent of the sheet. Values for T^* are: a, ∞ ; b, 30,000 yr; c, 10,000 yr; d, 5,000 yr.

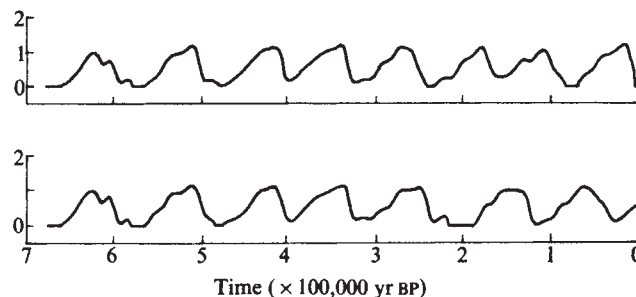


Fig. 4 Two model runs in which the Milankovitch insolation variations are imposed. The vertical axis gives a measure of the ice volume; a value of 1 on the scale corresponds to a meridional ice-sheet size of about 2,000 km and a maximum ice thickness of about 3,000 m. For more details on the parameterization of the mass balance see ref. 9. The only difference between the two runs is the value of T^* , 10,000 and 11,000 yr, respectively, in the upper and lower runs.

bedrock brings a large part of the ice surface beneath the equilibrium line, thus setting the breakdown in motion. Here, significantly, a sawtooth shape appears.

Figure 3d shows the situation when $T^* = 5,000$ yr, so that the isostatic balance is restored very quickly. In this case, the ice sheet cannot grow—the bedrock sinking completely cancels the effect of the height-mass balance feedback.

I next examined the situation in which the model is forced by real insolation variations. These variations were computed from expansions of the orbital parameters given by Berger¹⁰ and translated into mass-balance variations by using an ice/snow melt model⁹. In each of two runs performed, the integration was started at 675,000 yr BP, with initial condition $H(x) = h(x) = 0$.

Figure 4 shows the results. The only difference between the two runs is the value used for the time scale of isostatic adjustment. The agreement in the character of the simulated curves and the $\delta^{18}\text{O}$ record is striking—a 100,000-yr periodicity and an asymmetric shape of the glacial cycles. However, a different situation is obtained with regard to the phase of the cycles. Although dating of the $\delta^{18}\text{O}$ record is in fact not accurate enough to verify the model simulation of all glacial cycles, a comparison of the curves shown in Fig. 4 indicates that the phase of the glacial cycles seems to be extremely sensitive to the model parameters. I believe that this property of the model reflects a realistic feature: the global climatic variations of the Pleistocene seem to have been of a stochastic periodic nature. In view of this, it seems unlikely that the present model can produce an 'in-phase simulation' of the observed ice-volume record, but it does provide a physical explanation for the occurrence of the glacial cycles.

One of the implications of the present results is that the 100,000-yr cycle is mainly internally generated by the interaction of the bedrock adjustment and the height-mass balance feedback. This means that the occurrence of a 100,000-yr peak in the power spectrum of the $\delta^{18}\text{O}$ record² is unrelated to variations in eccentricity. Recent work by Kominz and Pisias¹¹ strongly supports this view: they found that coherence spectra of an oxygen isotope record and the orbital parameters showed no significant peak around a period of 100,000 yr.

Because the present theory deals with two large ice sheets on the Northern Hemisphere continents, it seems unlikely that they can be simulated with exactly the same model parameters. It is possible, however, that the Laurentide ice sheet dominates, and trails the Eurasian one with it through the atmospheric/oceanic teleconnection. Another objection to the theory is the passive role of the Southern Hemisphere. I must stress that the fact that global ice volume seems to lag the Southern Hemisphere sea-ice extent or sea-surface temperature² need not imply that events in the Southern Hemisphere initiate glacial cycles. This may be simply a matter of different response times.

Thus, my main conclusion is that the 100,000-yr cycle can be explained by ice sheet/Earth's crust dynamics, as was suggested by Weertman⁴. Due to bedrock sinking, ice sheets grow very slowly. If the bedrock sinking becomes more substantial, a larger part of the ice surface comes below the equilibrium line, setting the breakdown in motion. Another glacial cycle starts if two conditions are fulfilled—summer insolation should be well below 'normal' and the bedrock should have been raised again.

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Carbon-13 in tree-ring cellulose as an indicator of past climates

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Following Urey's suggestion¹ that the ^{13}C abundance (usually expressed as a relative ratio: $^{13}\text{C}/^{12}\text{C}$ or $\delta^{13}\text{C}$) fixed in plant carbon may be temperature dependent, several authors have investigated the relationship between $\delta^{13}\text{C}$ and climate. These workers searched for a coefficient relating $\delta^{13}\text{C}$ variations to temperature variations^{2–7}. The results, obtained on field collected samples, failed to yield agreement about the extent of a climatic effect or even its existence. Growth chamber experiments, performed on different plant species in controlled conditions to eliminate environmental factors other than temperature, showed no clear relationship between $\delta^{13}\text{C}$ and temperature^{8,9}. These various contradictory results suggest that factors other than temperature contribute to determining $\delta^{13}\text{C}$ in plants. We now present evidence that the variations of the relative abundance of stable isotopes of carbon in tree rings indicate past climate changes. Thus carbon isotope composition appears to be a proxy indicator that may help to extend the meteorological data base into pre-instrument times.

To gain a better understanding of the relationship between $\delta^{13}\text{C}$ values in tree rings and climate, we analysed two dendrochronologically-dated tree sections collected at Chetro Ketl, an archaeological site in Chaco Canyon, New Mexico. These samples, one, of ponderosa pine (*Pinus ponderosa*) dating from AD 810 to 1045 (sample CK-713), the other, a white fir (*Abies concolor*) dating from AD 931 to 1047 (sample CK-857), were provided by the Laboratory of Tree-Ring Research at the University of Arizona. Tree-ring material was chosen for the experiments because it can be absolutely dated, and, in this case, tree-ring width variations provided information on climate variations with which to correlate our isotope data. These variations are derived from the regional tree-ring chronologies for Chaco Canyon, of which there is one for ponderosa pine and one for white fir^{10,11}. The dendrochronological record is obtained by standardizing the ring widths of each one of several trees in one region, forming ring-width indices, with a mean value of 1.0 (see refs 10–12). These indices are averaged over the several individual trees to obtain the regional tree-ring chronology, which is given as a time series of mean standardized ring-width indices^{12–14}. This filters the growth effects experienced by individual trees, while preserving climatically induced varia-

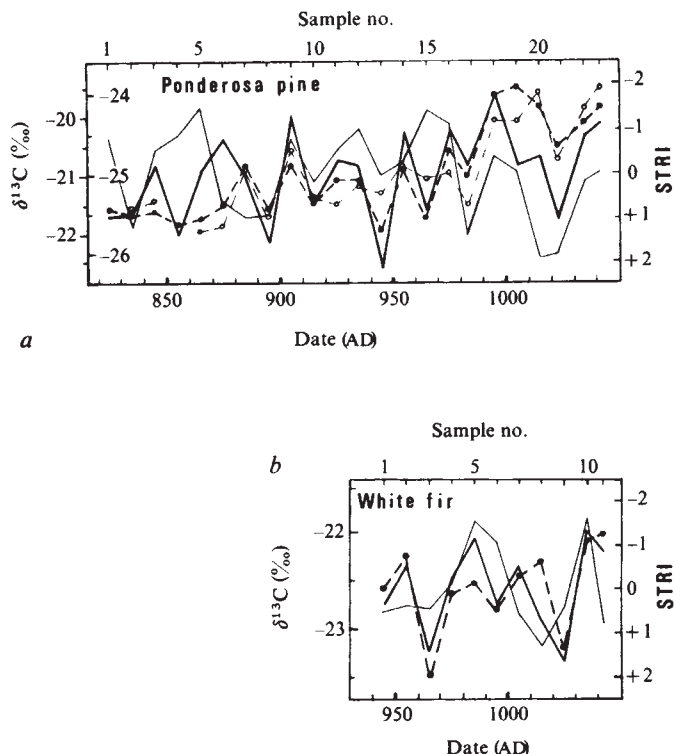


Fig. 1 Time series $\delta^{13}\text{C}$ values and tree-ring indices in the three sections from the Chetro Ketl site. All $\delta^{13}\text{C}$ values are expressed by reference to the PDB standard. The $\delta^{13}\text{C}$ scales and the STRI (standard tree ring index) scales have been normalized by subtracting to each value in a series the mean of each series then dividing it by the corresponding standard deviation. *a*, Thick line: decadal means of the Chaco Canyon regional ponderosa mean STRI. Thin line: decadal means of the individual STRI for the sampled section. Open circles: $\delta^{13}\text{C}$ (lignin) decadal values (inner scale). Solid circles: $\delta^{13}\text{C}$ (cellulose) decadal values (outer scale). *b*, Thick line: decadal means of the Chaco Canyon regional white fir mean STRI. Thin line: decadal means of the individual STRI for the samples section. Solid circles: $\delta^{13}\text{C}$ (cellulose) decadal values.

tions. In the Chaco Canyon and similar arid areas, ring-widths generally relate to climate in the following way: wide rings are produced during wet-cool years, and narrow rings during warm-dry years^{14–16}. The strength of these qualitative relationships has been discussed in statistical terms in recent work by Fritts *et al.*¹⁷.

Before we could ascertain the significance of a time series of carbon isotope values and test the potential of this method for the reconstruction of palaeoclimates, we had first to determine which chemical component of wood—cellulose or lignin—is most sensitive to climate; and second, to assess the uniformity of the isotope record by studying the δ values in different tree species from the same site and by measuring the variations in isotope values of a single set of rings around the tree circumference. We began our experiment by sampling a single radius of the ponderosa pine section. This provided 22 samples of 10 yr each and one of 6 yr, beginning at AD 820. These and later samples were ground into sawdust and extracted with benzene and ethanol in a Soxhlet extractor to remove oils, waxes, resins and other relatively mobile materials which may not be coeval with the corresponding tree ring. The extracted wood was separated into cellulose and lignin using standard chemical procedures similar to those described by Browning¹⁸. Carbon dioxide obtained from combustion of lignin or cellulose at 800 °C was analysed with an isotope ratio mass spectrometer (Micromass 602C) with an overall precision better than 0.1‰. The results are shown in Table 1 and in Figs 1a and 2a. (In the discussion that follows we give the correlation coefficients r as well as their values adjusted to remove the trends reflected by the first-order autocorrelations. These adjusted values are given

within square brackets. The adjustment was done by transforming the values in each measured series using the formula given in ref. 17, p. 21. The figures in parentheses are the 95% confidence limits.)

Results from this analysis show that the $\delta^{13}\text{C}$ values of cellulose and lignin strongly correlate with each other, $r = 0.914$ (0.80 to 0.96) [0.858 (0.65 to 0.95)] (Fig. 2a). The cellulose, however, has a higher correlation coefficient, $r = -0.653$ (-0.33 to -0.84) [-0.837 (-0.64 to -0.93)] with the ponderosa pine regional tree-ring index than does the lignin $r = -0.497$ (-0.08 to -0.76) [-0.666 (-0.29 to -0.86)] (Figs 1a, 2a, b). Thus, in our second experiment, on white fir samples, we analysed only the cellulose. The white fir section gave 11 samples dated between AD 940 and 1045, coeval with the outer 11 ponderosa pine samples. The $\delta^{13}\text{C}$ values for white fir are in general about 1.5‰ lower than the $\delta^{13}\text{C}$ values in ponderosa pine and also correlate well with the white fir regional tree-ring indices $r = -0.800$ (-0.38 to -0.95) [-0.761 (-0.25 to -0.94)] (Figs 1b and 2c).

Studies by other investigators have shown circumferential variations of $\delta^{13}\text{C}$ within a tree ring^{19,20}. To check for the variations of $\delta^{13}\text{C}$ values for different azimuths around our ponderosa pine tree section and their effect on our results we analysed three different sets of rings from four radial cuts other than the original radius samples. Only cellulose was isolated. The results on different radii show circumferential variations of an amplitude of about 1‰, but the relative variations observed between the different sets of rings are almost identical (Fig. 3). However, a sequence of average isotope values composed of $\delta^{13}\text{C}$ values from several radii reflects the tree-ring width variations more closely than $\delta^{13}\text{C}$ values from a single radius (for example, the one at azimuth = 0°) does.

Our results suggest a valid correlation between carbon isotope values and climate. The $\delta^{13}\text{C}$ values for both cellulose and lignin reflect the climatic variations recorded by the regional tree-ring width indices. Of the two wood components, cellulose seems to be the most sensitive to climate. Two facts argue against this relationship being an artefact of ring width as Wigley *et al.*²¹ recently suggested. First, our isotope values, which have been measured on each of the sections (of different species), correlate

with the regional climatic regime recorded by the regional tree-ring index and, second but most important, the isotope values do not correlate with the tree-ring indices derived from the measurements on the single tree from which the isotope samples were taken. The correlation coefficients between $\delta^{13}\text{C}$ and tree-ring indices for each one of our specimens are not significant at the 95% level: $r = 0.256$ (-0.17 to 0.60) [-0.167 (-0.55 to 0.27)] for the ponderosa pine, and -0.072 (-0.64 to 0.55) [0.065 (-0.59 to 0.67)] for the white fir. This indicates that the carbon isotopes reflect mainly regional conditions and are not largely dependent on factors which affect only individual trees, such as tree growth or other metabolic factors. A source of systematic error might be the fact that samples containing groups of rings instead of individual rings are weighted averages of wood (cellulose), thus the δ -values are not the average values for 10 individual years. In our samples the actual ring widths of the ponderosa pine and white fir sections varied by $\pm 30\%$, therefore, we do not expect that the method introduced large systematic errors. A precise estimate of the weighting error would require the knowledge of a well defined coefficient (δ -value)/(ring-width). Since such a coefficient could only be determined on the basis of many $\delta^{13}\text{C}$ analyses of individual single-year tree-rings, we could in our case, only roughly estimate it and we conclude that its magnitude did not affect our interpretation of the analyses. Obviously, single ring samples are to be preferred for palaeoclimatic research.

Several mechanisms could contribute to the observed relationship between $\delta^{13}\text{C}$ and climate. A simple but still speculative one, has been described elsewhere²². It is based on the fact that $\delta^{13}\text{C}$ in plant cellulose depends on the $\delta^{13}\text{C}$ value of the atmospheric CO_2 which surrounds the leaves during photosynthesis. The atmospheric $\delta^{13}\text{C}$ values vary diurnally as well as seasonally, and are more negative in times and places of greater plant respiration²²⁻²⁵ because CO_2 contributed by plant respiration is depleted in $\delta^{13}\text{C}$ (for example, causing plants grown in the recirculated respired CO_2 atmosphere of very dense forests to have a δ -value as low as -39‰) (see refs 22-25). Consequently, during times when climate favours greater vegetation activity, the local atmosphere $\delta^{13}\text{C}$ values would become more negative, a change reflected in the carbon fixed in the plant cellulose^{24,25}.

Table 1 Cellulose and lignin contents of tree-ring samples

<i>Pinus ponderosa</i> : sample CK-713					<i>Abies concolor</i> : sample CK-857		
Sample date (AD)	Regional index	Specimen index	Cellulose $\delta^{13}\text{C}$ (‰)	Lignin $\delta^{13}\text{C}$ (‰)	Regional index	Specimen index	Cellulose $\delta^{13}\text{C}$ (‰)
820-829	1.178	0.767	-21.60	—	—	—	—
830-839	1.166	1.434	-21.69	-25.43	—	—	—
840-849	0.957	0.844	-21.63	-25.34	—	—	—
850-859	1.259	0.733	-21.85	—	—	—	—
860-869	0.983	0.531	-21.73	-25.69	—	—	—
870-879	0.851	1.234	-21.53	-25.66	—	—	—
880-889	1.010	1.346	-20.83	-24.94	—	—	—
890-899	1.283	1.336	-21.57	-25.56	—	—	—
900-909	0.740	0.768	-20.82	-24.74	—	—	—
910-919	1.081	1.072	-21.45	-25.29	—	—	—
920-929	0.933	0.850	-21.05	-25.37	—	—	—
930-939	0.953	0.686	-21.05	-25.16	—	—	—
940-949	1.420	1.059	-21.95	-25.24	1.0399	1.1293	-22.59
950-959	0.823	0.919	-20.88	-24.91	0.9459	1.1049	-22.22
960-969	1.159	0.556	-21.72	-25.59	1.1498	1.1094	-23.52
970-979	0.808	0.656	-20.60	-25.02	0.9806	1.0013	-22.64
980-989	0.961	1.486	-20.99	-25.38	0.8761	0.6741	-22.50
990-999	0.659	0.898	-19.67	-24.36	1.0415	0.7891	-22.81
1000-1009	0.953	1.046	-19.50	-24.38	0.9460	1.1447	-22.46
1010-1019	0.924	1.666	-19.84	-24.03	1.0803	1.2953	-22.28
1020-1029	1.180	1.636	-20.51	-24.83	1.1723	1.0998	-23.20
1030-1039	0.831	1.101	-20.08	-24.22	0.8637	0.6740	-22.06
1040-1045	0.778	1.015	-19.82	-24.00	0.9115	1.1737	-22.00

No other factors, such as biochemical temperature effects, are necessary to explain the results of our analyses. The time series of Fig. 1a also shows an increasing trend in δ with time. This can probably also be ascribed to the local atmosphere existing near the soil, which is fixed by the young tree during its growth^{3,5}. The relationship described above between vegetation activity and local atmosphere²² explains the results of our research on both trees from the Chaco Canyon area (Fig. 1): increased local plant activity produces, (1) wider tree-rings, (2) more negative $\delta^{13}\text{C}$ values of the local atmosphere CO_2 , and, consequently more negative $\delta^{13}\text{C}$ of plant cellulose. Experimental field work on living junipers was done by Arnold²⁶. He compared the $\delta^{13}\text{C}$ in the leaves with records of close meteorological stations, and found that juniper leaves in a wide area of Arizona have more negative δ -values for years when the temperature during their growth was low and the precipitation high. These conditions are precisely those which determine wide growth rings for trees in these arid areas¹²⁻¹⁴.

We should also discuss an apparent contradiction between the results of the present analysis and the model²². The model seems to require that trees from the same region should have identical $\delta^{13}\text{C}$ values, but we evaluated the correlation coefficient between the $\delta^{13}\text{C}$ values in the cellulose of the ponderosa pine and white fir samples with each other and found it to be only 0.454 (-0.20 to 0.83) [0.518 (-0.17 to 0.87)]. At first this seems

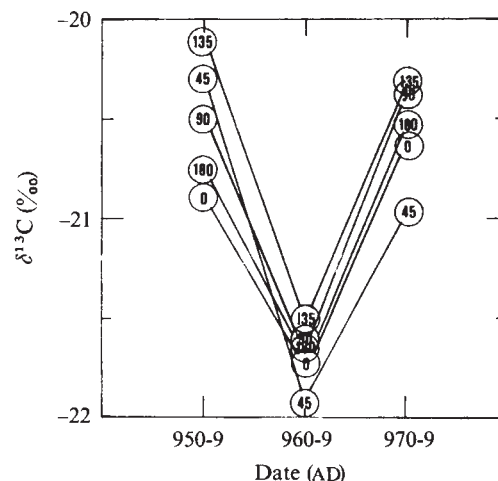


Fig. 3 Radial series: $\delta^{13}\text{C}$ (cellulose) in the ponderosa pine section at 5 different radii for 3 different decades. The figures indicate the azimuth in degrees. The azimuth 0° samples correspond to the time series of Fig. 1a.

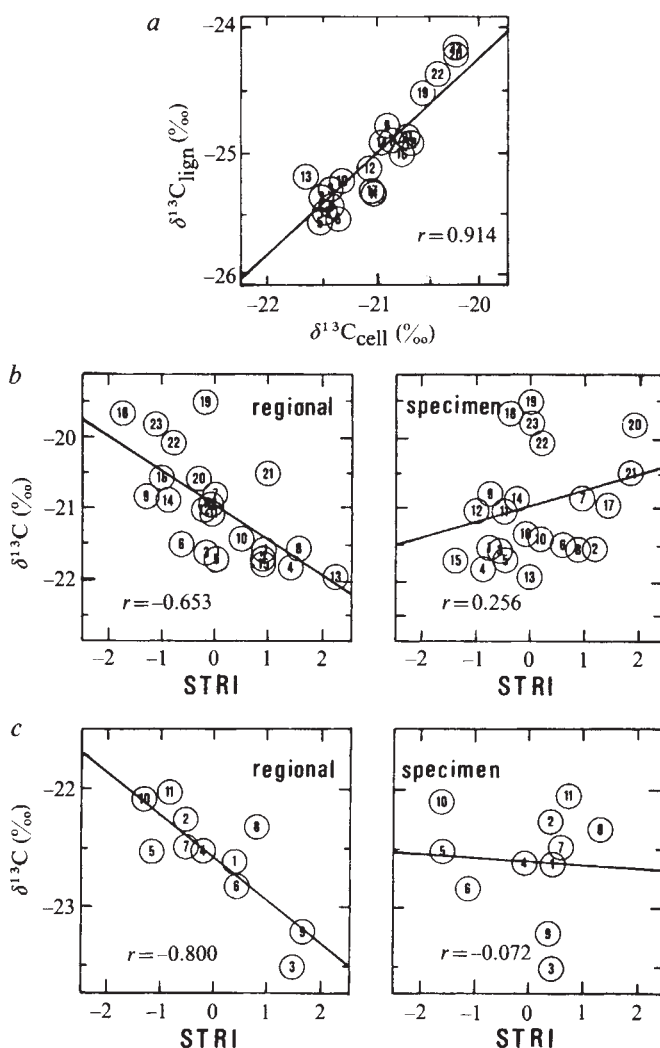


Fig. 2 Linear regressions (the figures within the circles represent sample numbers as given in Fig. 1). The r values adjusted for autocorrelations of first order are given in the text. a, $\delta^{13}\text{C}$ (cellulose) versus $\delta^{13}\text{C}$ (lignin) in ponderosa pine samples. b, $\delta^{13}\text{C}$ (cellulose) in ponderosa pine samples versus regional STRI (standardized tree-ring indices) for ponderosa pine. c, $\delta^{13}\text{C}$ (cellulose) in white fir samples versus regional STRI for white fir.

to conflict with a model hypothesizing atmospheric CO_2 fluctuations as the mechanism for $\delta^{13}\text{C}$ fluctuations observed in tree rings. The explanation for this rather low correlation between $\delta^{13}\text{C}$ values of our two trees from the same region is that these trees inhabit different ecological niches which would have different local atmospheres. Ponderosa pine is found in warm and dry environments while white fir is partial to cooler and wetter environments²⁷. More plant growth occurs in the wetter white fir environment, thus CO_2 more locally respired by white fir trees and other plants contributes to a more negative $\delta^{13}\text{C}$ value for the local atmosphere CO_2 . This would not be in equilibrium with the bulk atmosphere, particularly in the early morning, as our field measurements showed for the White Mountains, California²². The white fir $\delta^{13}\text{C}$ values reflect this more negative value. This also may account for the absence of large positive peaks in the $\delta^{13}\text{C}$ white fir data, peaks which are present in the ponderosa pine data (Fig. 1a, b). This could explain the lack of a good correlation between $\delta^{13}\text{C}$ of both trees, while the lack of good correlation between both regional tree-ring chronologies would be due to each species having a different response^{14,15} to climate.

We have demonstrated a relationship between climate and stable carbon isotope values in tree rings. Cellulose is the most sensitive wood component, and in a single radius the time series variation are representative of climate. Stable carbon isotope analysis in tree rings may thus become an important method for extracting climate data from 'complacent' trees, those in which ring width variations are not responsive to climatic conditions (for example, trees tapping a permanent water source). In such a case, the isotope values of the wood should still record changes in the $\delta^{13}\text{C}$ values of the atmospheric CO_2 produced by fluctuations of the regional plant biomass. Moreover, from the above results, it seems that a single tree contains climate data equivalent to conventional dendroclimatology which, instead, uses the mean of several trees. Isotope dendroclimatology may thus be the proxy indicator that will help enlarge our data base for large scale climate models or simulations, and be also of benefit to archaeologists for local palaeoclimate reconstructions²⁸.

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Electrophoretic evidence for two species of *Anguilla* leptocephali in the Sargasso Sea

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Schmidt^{1,2} produced indirect evidence that in order to reproduce the European eel (*Anguilla anguilla*) migrates to a definite zone of the Sargasso Sea, contiguous and partially overlapping the breeding area of the American eel (*Anguilla rostrata*). The two forms were recognized as distinct species on the basis of differences in meristic characters^{3,4}. Later, Tucker⁵ proposed that the two Atlantic eel populations originate from the same gene pool and are not distinct species but merely eco-phenotypes, their distinguishing characters being environmentally determined. More recent electrophoretic analysis has favoured the existence of two distinct species^{6–9} and of particular significance from a genetic point of view is the very marked difference in malate dehydrogenase (MDH) allele frequencies between samples of American and European eels (refs 8, 10–12, Table 1a). However, inferring genetic isolation from genetic differentiation in eels is difficult^{13,14}, because of the possibility of differential selection. We report here the first observations on variability at four polymorphic loci in young *Anguilla* larvae caught in the breeding area. The observed genotype distribution and allele frequencies, particularly at the MDH-2 locus, definitely confirm the presence of two species of *Anguilla* leptocephali in the spawning zone of the Sargasso Sea.

The aim of the cruise of the FRV 'Anton Dohrn', from 19 March (Bermuda) to 9 May (Bremerhaven) (1979), was to resolve problems of sampling technique and egg and larval identification. During the period 21 March to 15 April, in the middle of spawning^{1,4}, more than 1,800 leptocephali of *Anguilla* were captured in different stations between 22–29° N and 55–70° W, in and near the spawning areas defined by Schmidt^{1,2}. As expected, the captured leptocephali were mainly of the O-group (first year larvae) 7–25 mm in length and no more than a few

Table 1 Observed (and expected) genotype distribution at the MDH-2 locus (see Fig. 1) and relative allele numbers and frequencies in European and American eels (a) and *Anguilla* leptocephali from the Sargasso Sea (b)

(a)				
MDH-2 genotypes	<i>A. anguilla</i> *		<i>A. rostrata</i> †	
aa	3	(1.6)	639	(638.2)
ac	65	(65.8)	55	(56.5)
cc	671	(679.1)	2	(1.3)
Others‡	340	(332.5)	—	—
Total	1,079		696	
Alleles	No.	Frequencies	No.	Frequencies
a	83	0.039	1,333	0.958
c	1,712	0.793	59	0.042
Others§	363	0.168	—	—
(b)				
Genotypes	No.	Alleles	No.	Frequencies
aa	72 (45.8)	a	152	0.603
ac	8 (53.1)	b	5	0.020
cc	35 (15.4)	c	88	0.349
Others	11 (11.7)	d	2	0.008
		e	5	0.020
Total	126		252	

Only genotypes and alleles common to both species are reported in full.

* Totals are obtained by pooling unpublished genotype distributions observed in samples of *A. anguilla*^{11,12}.

† Pooled data from Williams *et al.*¹⁰.

‡ Observed number of other genotypes also fitted well with Hardy-Weinberg distribution.

§ Frequencies of 'other' alleles: $b = 0.047$; $d = 0.025$; $e = 0.088$; three very rare alleles = 0.009.

|| 'Other' observed genotypes: $bb = 1$; $bc = 3$; $cd = 2$; $ce = 5$.

For (b), $\chi^2_{(3)} = 78.3$, $P < 0.01$.

weeks old⁴. Details of the fishing technique, place of capture and larval distribution are reported elsewhere¹⁵. Most of the larvae were preserved for myomere counts in the laboratory; 121 individuals of the O-group and five of the I-group (about 40 mm) were analysed on board by starch gel electrophoresis. Myomere counting on board was very rough due to local conditions and was later shown to be not completely reliable (for details, see ref. 16).

Among enzyme systems reported polymorphic in elvers and older eels^{8,10–12}, only three showed enough activity or clear enough electrophoretic patterns for a reliable genetic interpretation—MDH, phosphoglucose isomerase (PGI) and lactate dehydrogenase (LDH). Special attention was given to the scoring of MDH because of the previously reported difference in allelic frequencies between the two populations.

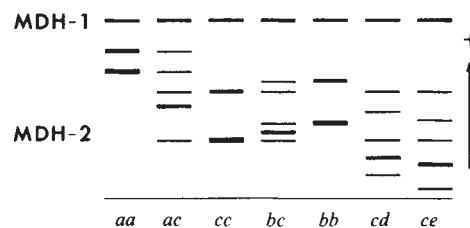


Fig. 1 Schematic representation of the MDH electrophoretic patterns observed in the leptocephali sample and genetic interpretation of variability at the MDH-2 locus. It is assumed that polypeptide products of the two loci randomly assemble to form homodimers and intralocus and interlocus heterodimers of intermediate electrophoretic mobility. Following our previous convention¹⁷, the alleles are indicated by a, b, c, d, e in the order of the anodic mobility of the respective homodimer bands. Previous laboratory analysis of the MDH pattern in a sample of American eels has shown that the common MDH-2 allele of *A. rostrata* (named a by Williams *et al.*¹⁰) has the same electrophoretic mobility as the fast allele a previously reported in *A. anguilla*^{11,12,17}; the rare allele b of *A. rostrata*¹⁰ corresponds to the common allele c of *A. anguilla*^{11,12,17}.

Table 2 Observed (and expected) genotype distribution at PGI-1, PGI-2 and LDH-2 loci in samples of *A. anguilla* and *A. rostrata* leptocephali.

Loci and genotypes*	<i>A. anguilla</i> †	<i>A. rostrata</i> †
PGI-1		
<i>bb</i>	28 (29.8)	67 (67.1)
<i>ab</i>	9 (7.2)	4 (3.9)
<i>bc</i>	9 (7.2)	1 (0.9)
Others	0 (1.8)	0 (0.1)
Total	46	72
	‡ <i>P</i> < 0.001	
PGI-2		
<i>bb</i>	39 (39.3)	67 (67.1)
<i>ab</i>	2 (1.8)	3 (2.9)
<i>bc</i>	4 (3.7)	2 (1.9)
<i>bd</i>	1 (0.9)	0 (0.0)
Others	0 (0.3)	0 (0.1)
Total	46	72
	n.s.	
LDH-2		
<i>aa</i>	29 (29.4)	50 (50.0)
<i>bb</i>	0 (0.3)	0 (0.0)
<i>ab</i>	7 (6.3)	1 (1.0)
Total	36	51
	<i>P</i> < 0.01	

* The genetic interpretation of the electrophoretic patterns and the electrophoretic methods are reported in refs 11, 17, 20.

† Species identification on the basis of MDH-2 genotypes.

‡ P values are computed with contingency χ^2 tests on the number of alleles, and with a weighted homogeneity test for allele frequencies²¹.

The larval MDH electrophoretic patterns (Fig. 1) correspond closely to those previously described in elvers and adults of *A. anguilla* (Fig. 2) and *A. rostrata*¹⁰; as in other teleosts¹⁸ they are genetically explicable assuming the presence of two MDH loci, one of which is polymorphic in the *Anguilla* species. The observed and expected genotype distribution and the allele frequencies for the polymorphic locus MDH-2, in the whole larval sample, are reported in Table 1b. The observed genotype distribution shows a very strong homozygote excess as compared with that expected according to the Hardy-Weinberg law.

Excluding inbreeding and selection against heterozygotes in a sample consisting mostly of very young larvae, the homozygote excess most probably results from the sampling together of individuals originating from two distinct populations with appreciable difference in allele frequencies (the Wahlund effect). This is consistent with the view that the sample was made up of groups of larvae belonging to both *Anguilla* species having respectively the same, or very similar, allele frequencies to those reported for samples of elvers and older eels 'recruits' and 'residents', respectively¹⁰.

If this is true, then the MDH-2 genotype can also be useful in young leptocephali as a diagnostic character of the species, easier to recognize and more reliable at this stage than the rough myomere count made on board^{15,16}. Indeed, in pooled samples of recruits and residents, on the basis of the reported MDH-2 allele frequencies (Table 1a), the probability of correct diagnosis of the species is more than 0.998 (as calculated applying the Ayala and Powell¹⁹ statistics) if the aa homozygotes are scored as *A. rostrata*, the heterozygotes showing the 'ambiguous' genotype ac are not considered and all the other genotypes are scored as *A. anguilla*.

With the use of this parameter and omitting the eight observed ac individuals, the remaining 118 leptocephali analysed on board can be separated into 72 *A. rostrata* (aa genotypes) and 46 *A. anguilla* (cc and other genotypes). Laboratory myomere counts of preserved larvae, now in progress, are indeed showing that in the main batch of leptocephali *A. rostrata* larvae were the more abundant (F.-W. Tesch, personal communication).

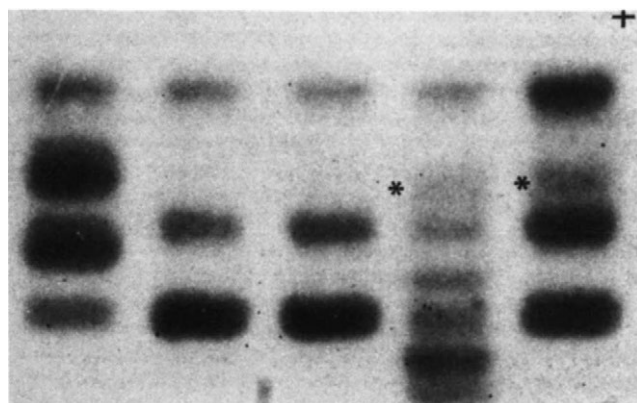


Fig. 2 Photograph of some MDH electrophoretic patterns observed in specimens of *A. anguilla*. The MDH-2 genotypes, from left to right, are: ac, cc, cc, cc, cc. Asterisks indicate bands corresponding to the mitochondrial MDH activity which appear in frozen specimens. Horizontal electrophoresis in 11.5% starch gel was carried out using electrode buffer 0.18 M Tris-citrate, pH 7.5, 1:15 diluted in the gel. The staining solution was: neutralized L-malic acid (10^{-2} M), NAD (10^{-3} M), monotetrazolium salt (10^{-3} M), phenazine methosulphate (10^{-4} M) in 0.05 M Tris-HCl, pH 8.

On the basis of the previous assumption, we report in Table 2 the genotype distribution at the PGI-1, PGI-2 and LDH-2 loci for the two larval groups. The observed numbers are in each case near to the expected assuming Hardy-Weinberg equilibrium. Comparison of allele numbers and of allele frequencies with statistical tests showed significant differences at the PGI-1 and LDH-2 loci, supporting the genetic separation of the two groups. Table 3 shows the allele frequencies at the same loci for both groups, in comparison with those observed in pooled samples of *A. anguilla*^{11,12} and *A. rostrata*¹⁴, respectively.

In *A. anguilla*, there is no appreciable difference, at these loci, between larvae caught in the spawning zone and eels caught in different European coastal waters¹¹. It seems, therefore, that there is no differential selection at these loci during the years of larval drift; this is in accord with our previous observations of a lack of genetic differentiation among samples of recruits from different parts of Europe^{11,12,20}.

Table 3 Allele frequencies at PGI-1, PGI-2 and LDH-2 loci in samples of *A. anguilla* and *A. rostrata* leptocephali (L). For comparison, data from pooled samples (P) of both eel species are reported.

Loci and alleles	<i>A. anguilla</i>		<i>A. rostrata</i>	
	L	P*	L	P†
PGI-1				
<i>a</i>	46	1,058	72	
<i>b</i>	0.098	0.083	0.028	
<i>c</i>	0.804	0.822	0.965	No
Others	0.098	0.089	0.007	data
	—	0.006	—	
	n.s.			
PGI-2				
<i>a</i>	46	1,067	72	2,271
<i>b</i>	0.022	0.008	0.021	0.009
<i>c</i>	0.924	0.941	0.965	0.788
<i>d</i>	0.043	0.049	0.014	0.203
	0.011	0.002	—	—
	n.s.		(‡ <i>P</i> < 0.001)	
LDH-2				
<i>a</i>	36	820	51	
<i>b</i>	0.903	0.915	0.990	No
	0.097	0.085	0.010	data
	n.s.			

* Pooled data from our previous paper^{11,12}.

† Pooled data for 'recruits' only from Koehn and Williams¹⁴.

‡ P values are computed according to Workman and Niswander test²¹.

In *A. rostrata*, comparable data are available only for the PGI-2 locus. There is a very significant difference in allele frequencies between the larval sample and that reported for recruits of the North American shore. Moreover, in resident eels of this species, a latitudinal cline in allele frequencies at this locus was observed, while recruits were genetically homogeneous¹⁰. This pattern was demonstrated to be temporally constant¹⁴. The higher frequency of the common allele *b* (>0.9, and close to that found in *leptocephali*) was obtained in the northern part of the sampled range (Halifax, Nova Scotia; St John's, Newfoundland). It is perhaps premature to make the hypothesis of a temporal or geographical subdivision in the spawning of *A. rostrata* adults coming from different parts of the American coast; however, we note that some doubts still remain about the extension of the spawning place of this species²².

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In conclusion, observation on the genetic variability of some enzyme loci in a sample of young *Anguilla leptocephali* caught in the spawning ground have shown the existence in this zone of larvae from two different gene pools, thus definitively confirming Schmidt's theory of the existence of two distinct Atlantic *Anguilla* species.

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Cytolytically active murine T-cell hybrids

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Following the demonstration that hybrids between normal B-lymphocytes and myeloma cell lines continue to secrete antibodies with the same specificity as those produced by the parental B-cells¹, many groups have tried to use this approach to obtain cell lines expressing T-lymphocyte functions by crossing thymoma lines not expressing any measurable activity with various types of T-cell populations. Although there have been reports that hybrids could be isolated which secrete T-cell products with immunological activity^{2–5}, efforts to produce functionally active hybrids from cytolytic T-cells have all been unsuccessful (refs 6, 7, and M. N. and H. D. Engers, unpublished). We have fused an established, T-cell growth factor (TCGF)-dependent murine cytolytic T-lymphocyte (CTL) line with a mouse thymoma line and have obtained hybrids with cytolytic activity when we selected the hybrids in TCGF-containing medium, while hybrids isolated in the absence of growth factor showed no detectable cytolytic potential.

Recently, clonable murine T-cell lines with cytolytic activity have been derived from normal CTL populations cultured in medium supplemented with TCGF-containing supernatant from concanavalin A (Con A)-stimulated spleen cells (CS)^{8,9}. Such CTL lines remain strictly dependent on CS medium for growth, but share many characteristics with permanent cell lines derived from other tissues¹⁰. B6.1.2 is a cloned CTL line

obtained from spleen cells of a female C57Bl/6 mouse activated against syngeneic male cells. At the time of its isolation, it was cytolytically active against lipopolysaccharide-activated spleen cells (LPS-blasts) from male mice carrying the H-2D^b allele as well as from H-2D^d mice of either sex¹¹. A mutant, TG14.OU5, was derived from this line, which is resistant to both 6-thioguanine (TG^r, 10^{−4} M) and ouabain (OU^r, 10^{−3} M), by two cycles of mutagenesis with ethyl methane sulphonate, followed by single-step selection with one of the drugs¹⁰. The resistant phenotype of this mutant has remained stable for 8 months of growth in the absence of either drug. The parental cells and the mutant show comparable lytic activity against the BALB/c myeloma S194, but both lines today display only marginal activity against LPS-blast targets.

B6.1.2 and TG14.OU5 have a near-diploid karyotype with several abnormal metacentric chromosomes¹⁰ (Table 1). The cells have an irregular shape and grow mainly in suspension with only a slight tendency to adhere (Fig. 1). CSP.2 is a CTL line derived from C3H/HeJ (H-2^k) cells activated against syngeneic spleen cells haptenated with 3-(p-sulphophenyldiazo)-4-hydroxyphenylacetic acid (SP)¹⁰. In its lytic activity this line is hapten specific and H-2 restricted (Fig. 4): it will lyse SP-coupled LPS-blasts from B10-A (H-2K^dD^d) mice but shows no significant activity against haptenated B10.AQR (H-2K^dD^d) target cells.

B6.1.2, TG14.OU5 and CSP.2 all express the Thy-1.2 alloantigen. The thymoma lines used in the experiments described here, AKR-A (from Dr R. Herberman, NCI) and a TG^rOU^r BW5147 subline (from Dr. R. Hyman, Salk Institute) express the Thy-1.1 allele. They grow in suspension, in medium with or without CS, and show no detectable lytic activity against S194 or LPS-blasts, even when Con A is added to overcome the requirement for specific target-cell recognition¹². AKR-A has a near-tetraploid karyotype and most cells carry one characteristic metacentric chromosome (Table 1).

Cells of the parent lines were mixed and fused, and plated out in different media (see Fig. 2 legend for details). Two to three weeks after fusion the plates were scored for growth: no growth was detected in plates with or without CS seeded with PEG-treated cells of only one of the parental lines or with untreated cell mixtures. In plates seeded with mixtures of AKR-A and TG14.OU5 cells treated with 50% polyethylene glycol

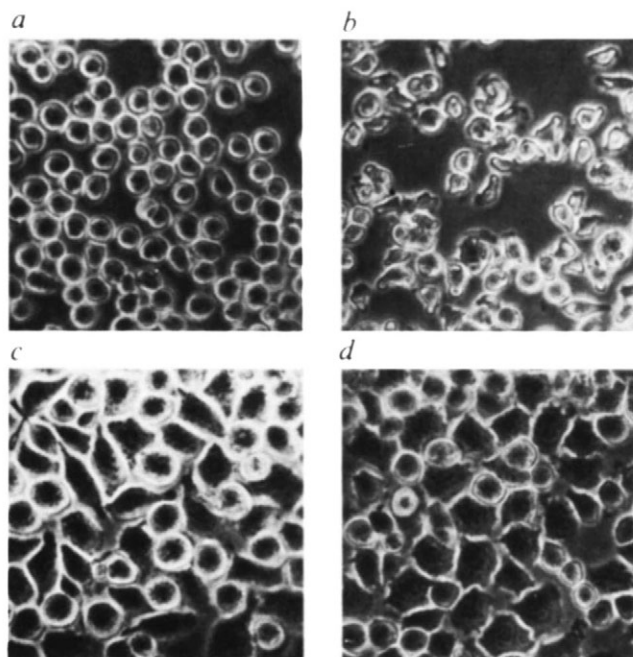


Fig. 1 Morphology of parental cell lines, AKR-A (a) and B6.1.2 TG14.OU5 (b) and two cytolytically active hybrid clones, MN9.19.7 (c) and MN9.20.2 (d). Phase-contrast micrographs of living cells.

(PEG), vigorous growth was observed in 70 of 96 wells not containing CS, and in 95 of 96 wells with CS. The cells growing in the absence of CS were round or slightly irregular and resembled the thymoma parent. They showed no tendency to adhere to the plastic surface and piled up to form regularly shaped colonies. The same cell type was found in many wells containing CS, but in addition most of the latter wells contained colonies formed by cells with a morphology quite distinct from that of either parent (Fig. 1): these cells were very large and flat and adhered quite strongly to the plastic surface. Size and shape varied somewhat from colony to colony, and although they clearly showed a strong tendency to adhere they could form multiple layers.

Table 1 Chromosome constitution of parental cells and two hybrid clones

	Total	Biarmed	Markers		
			AKR-A	TG14.OU5	
AKR-A	77.5(71-79) ±2.3	1.8(0-2) ±0.4	9/10	0/10	0/10
TG14.OU5	32.2(28-34) ±1.7	8.1(8-9) ±0.3	0/14	14/14	14/14
MN9.19.7	126.7(93-144) ±15.8	11.4(9-14) ±1.4	8/12	12/12	12/12
MN9.20.2	89.3(81-105) ±21.9	8.1(6/9) ±1.3	10/10	10/10	9/10

Chromosomes were prepared using the ASG-banding technique¹⁷ and classified according to Nesbitt and Franke¹⁸. Chromosome number (range) ± s.d. was determined and the same metaphases were screened for the presence of marker chromosomes characteristic for the parental cell lines. Almost all MN9.19.7 metaphases contained two copies of each of the TG14.OU5 markers. TG14.OU5 lacks recognizable chromosomes 6, 7 and 8, whilst AKR-A and the hybrids contain several copies of these chromosomes.

We found that none of 34 populations selected in the absence of CS displayed any cytolytic activity whereas 60 of 79 presumptive hybrids selected in the presence of CS gave more than 10% specific lysis in the presence of Con A (Fig. 2). When the morphology of the latter hybrids was scored, it became apparent that all of the populations from the six wells containing only or almost only thymoma-like cells gave less than 10% lysis. Ten independent (uncloned) populations, selected in the presence of CS, were expanded and maintained in CS medium containing HAT and ouabain (HOS medium). Their growth rate is comparable to that of the CTL parent line. Two of these populations consisted entirely of cells with the thymoma-like morphology. All 10 populations expressed both parental Thy-1 alleles: more than 90% of the cells were lysed when treated with monoclonal anti-Thy-1.2 or anti-Thy-1.1 antibodies and complement. Background lysis was less than 10%. The cytolytic activity of these hybrids against S194 varied (Fig. 3). The two thymoma-like hybrids had no detectable activity, even in the presence of Con A. The adherently growing hybrids all expressed some activity in the presence of the lectin and all except one showed some lysis also in its absence.

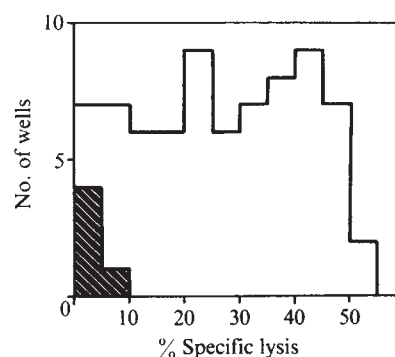


Fig. 2 Cytolytic activity of AKR-A × TG14.OU5 hybrids selected in medium supplemented with Con A supernatant. Before fusion the drug-resistant parents were grown for two passages in medium containing thioguanine and ouabain to eliminate any revertants. 1×10^7 cells of either parent were mixed and fused using 50% PEG (molecular weight 1,000) in a procedure essentially identical to the one described by Galfré *et al.*¹⁴. The fused cells were plated out at a density of 1×10^4 per cell type per well per 100 μ l in flat-bottom microtitre plates (96 wells, Costar) in MLC medium or in MLC medium supplemented with 30% CS⁸. 100 μ l of the same medium containing 10^{-4} M hypoxanthine, 5×10^{-7} M amethopterin, 1.6×10^{-5} M thymidine (HAT)¹⁵ and 2×10^{-3} ouabain were added to each well on the next day. (This medium selects for cells in which the codominant ouabain resistance marker of one parent is complemented with the wild-type hypoxanthine phosphoribosyl transferase gene of the other.) One week later about two-thirds of the medium was replaced, keeping the HAT and ouabain concentration constant. 24 days after fusion cells were detached by vigorous pipetting with a multichannel micropipette (Flow) and transferred to round-bottom 96-well microtitre plates (Greiner). They were washed once and 10^4 51 Cr-labelled S194 target cells and Con A (10μ g ml⁻¹ final concentration) were added, all in Dulbecco's minimal essential medium with 5% fetal bovine serum. Plates were spun for 1 min at 1,000 r.p.m. and incubated for 6 h at 37°C. Specific isotope release into the medium was determined according to Brunner *et al.*¹⁶. Spontaneous release was 12%. Hatched bars show activity of populations from wells containing only thymoma-like cells.

Several of the independent cytolytically active hybrids were cloned, by plating cells at limiting dilutions (0.3 cells per well in HOS medium on feeder layers of irradiated peritoneal C3H cells⁸). Two independent clones have now been maintained in continuous culture for three months and are still cytolytically active, although the activity of one of them has declined considerably. Seventy days after fusion these two clones were

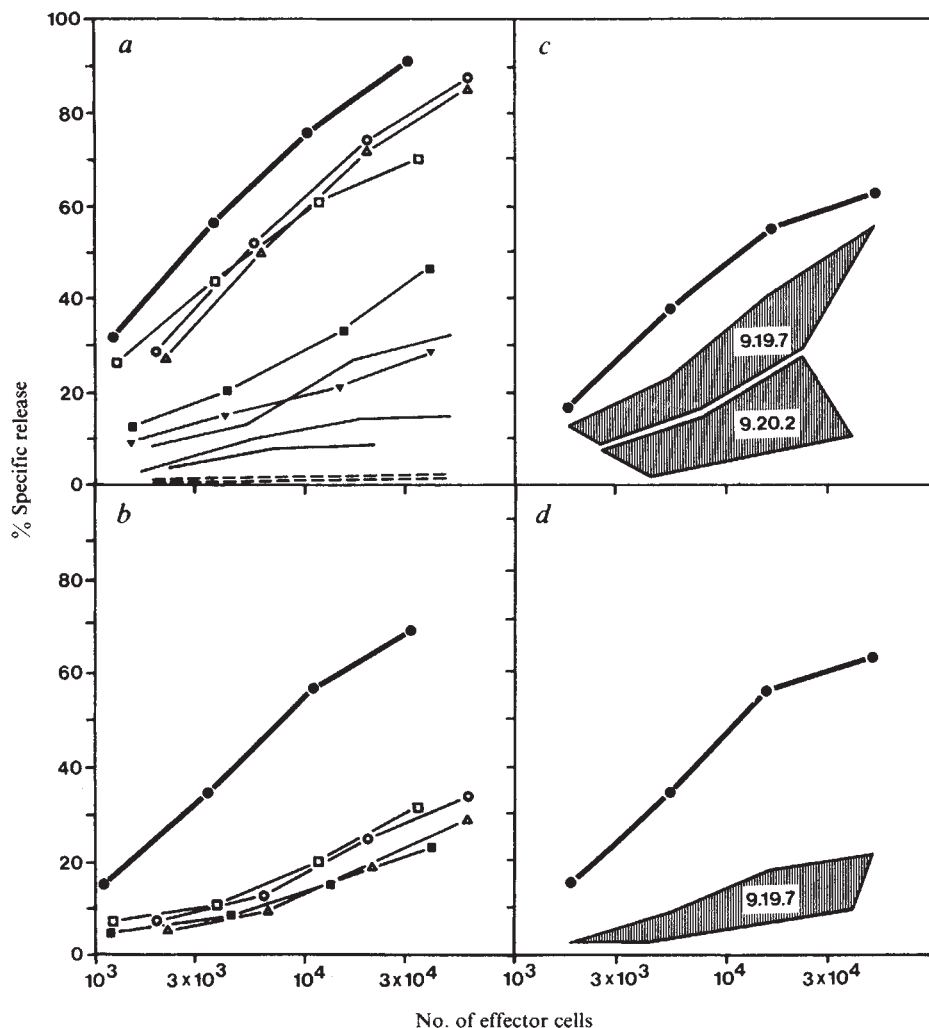


Fig. 3 Cytolytic activity (^{51}Cr release) of independent uncloned populations of AKR-A $\times$ TG14.OU5 hybrids (a, b) and individual clones (c, d), assayed in presence (a, c) and absence (b, d) of Con A. Targets: 10^4 S194 cells. Effector cells were collected during exponential growth. Adherent populations were detached with 0.02% EDTA in phosphate-buffered saline. $\bullet$, TG14.OU5. In a and b: —, adherently growing hybrids; ---, thymoma-like hybrids; $\blacksquare$, MN 9.19; $\blacktriangledown$, MN9.20. In c and d the shaded areas indicate the range of activities of subclones derived from two cloned hybrids: MN9.19.7 (five subclones) and MN9.20.2 (four subclones). In b and d only significant activities are plotted. All subclones of 9.19.7 were active.

subcloned. All of the tested subclones gave significant lysis when tested in the presence of Con A, but most of the clones derived from the weaker line had only marginal activity (Fig. 3). Karyotype analysis of these two lines demonstrates that one of them contains approximately one set of each of the parental chromosomes, whilst the other carries two sets of TG14.OU5 chromosomes and one from the thymoma parent (Table 1).

It is very difficult to determine whether the AKR-A $\times$ TG14.OU5 hybrids described above express determinant-specific recognition structures, because, in the absence of Con A, the CTL-line parent as well as the hybrids show significant lytic activity only against tumour cell targets and, as we have argued elsewhere¹⁰, such targets are not adequate for a specificity analysis of established CTL lines.

We therefore crossed the TG'OU' BW5147 thymoma line with CSP.2 using an essentially identical procedure. The results of this fusion were very similar to those of the AKR-A $\times$ TG14.OU5 cross¹³, except that the morphological differences between the hybrids selected in the presence and those selected in the absence of CS were less obvious. We tested four hybrid populations selected without CS and seven selected in the presence of CS for expression of parental Thy-1 alleles and specific cytolytic activity. More than 90% of the cells of all populations were lysed by both anti-Thy-1.1 and anti-Thy-1.2 antibodies and complement. None of the CS-independent populations showed any lytic activity whilst all of the populations selected in CS lysed SP-coupled but not unhaptenated H-2^k LPS-blasts. Several of the active hybrids were tested against targets of different H-2 haplotypes and their activity was

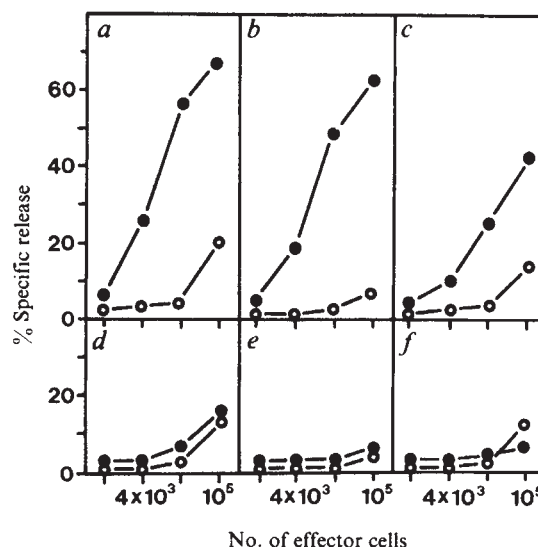


Fig. 4 Specificity and H-2 restriction of hybrids between BW5147 and CSP.2. Targets: LPS-blasts, untreated ($\circ$) or haptenated with 50 mM 3-(p-sulphophenyldiazo)-4-hydroxyphenylacetic acid ($\bullet$), from B10.A mice (a, b, c; H-2 haplotype K^kI-A^kI-C^dD^d) or B10.AQR (d, e, f; qkdd). Effector cells: a and d, CSP.2; b and e, hybrid MN12.18.17; c and f, hybrid MN12.7. 3-h assay with 10^4 targets, spontaneous release ranged from 13–15%.

found to be H-2 restricted (Fig. 4). Active clones were obtained by limiting dilution and have maintained their specific activity for several months of continuous culture.

Our findings suggest that the earlier failures to obtain cytolytically active CTL-thymoma hybrids were due to the fact that such hybrids share the requirement for TCGF with their CTL parents and were not detected because all selections were carried out in the absence of CS. However, alternative explanations are possible. Clearly, our results suggest a relationship between TCGF dependence and expression of cytolytic competence. But, at present, we cannot decide whether the cytolytically incompetent, TCGF-independent thymoma-like hybrids are derived from active, growth factor-dependent cells, for example, by segregation of a particular CTL-line chromosome or whether the active hybrids have lost a thymoma chromosome carrying a gene (or genes) suppressing the CTL phenotype in the inactive hybrids. Other, less attractive possibilities have to be envisaged: (1) the two types of hybrids may be

derived from different variants co-existing in the thymoma or the CTL lines. This interpretation is made unlikely by the very similar results obtained in three different crosses involving two thymomas and three CTL clones¹³. (2) Alternatively, the manifestation of the CTL phenotype in a hybrid may depend on a single stochastic event occurring early after fusion or it may be determined, for example, by the stage of the cell cycle in which the cells giving rise to a hybrid clone are at the moment of fusion.

Whatever the answers to these questions may be, it appears likely that cytolytic T-cell hybrids will provide us with new efficient tools for a genetic analysis of the control of the expression of T-cell functions.

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Control of natural resistance to *Salmonella typhimurium* and *Leishmania donovani* in mice by closely linked but distinct genetic loci

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Inbred strains of mice vary in their sensitivity to infection with both *Salmonella typhimurium*^{1,2} and *Leishmania donovani*³. In both cases, this differential susceptibility is genetically controlled. Resistance to the intracellular parasite *L. donovani* is determined by a single locus on chromosome 1, designated *Lsh* (ref. 4). The primary regulator of resistance to *S. typhimurium* is a single, dominant autosomal gene, named *Ity* (for immunity to *typhimurium*)⁵, and it has also been recently mapped to chromosome 1 (ref. 6). In addition, two other genetic loci regulate resistance to *S. typhimurium* in mice. These genes, *Lps*^a and *xld*, are mutant alleles that render C3H/HeJ and CBA/N mice, respectively, salmonella susceptible^{7,8}. Both Bradley and his colleagues^{3,4}, and Plant and Glynn^{2,6}, noted similar patterns of resistance or susceptibility of inbred strains of mice to *L. donovani* and *S. typhimurium*, and therefore suggested that *Lsh* and *Ity* might be the same gene. Mapping of both genes to the same region of chromosome 1 supported this hypothesis but no linkage studies have been used to test it. Since recombinant inbred (RI) mouse strains are, in effect, permanent segregant populations⁹, they are ideal for determining linkage between resistance genes to two different pathogens. Therefore, we determined the *S. typhimurium* susceptibility of five sets of RI mouse strains that had been previously typed for *Lsh*^a and conclude that *Lsh* and *Ity* are closely linked but distinct genetic loci.

Mice from 25 BXD RI and their salmonella-resistant (DBA/2J)⁵ and -susceptible (C57BL/6J)⁵ progenitor strains (Jackson Laboratory) were injected subcutaneously (s.c.) with approximately 25 *S. typhimurium* organisms of the virulent TML strain¹⁰. The actual number of organisms administered was determined by plate count. The cumulative 28-day mortality was then assessed. For each strain at least two separate experiments were performed and progenitor strain controls were always included. In addition, the geometric mean number of *S. typhimurium* per spleen for each RI strain was ascertained⁷ at day 9 after s.c. infection of mice with 10³ TML.

The number of viable organisms per spleen of animals which died from salmonellosis before day 9 was estimated as 10⁸ based on spleen counts in highly moribund mice. A comparison of the *Lsh* type, as previously reported by Bradley *et al.*, and the *Ity* types derived from our experiments, is shown in Table 1. RI strains were classified as *Ity*⁺ if ≥60% of the animals in a group died and if the log₁₀ geometric mean counts per spleen were >5.0, whereas strains were considered *Ity*⁺ if mortality was ≤40% and if the log₁₀ geometric mean counts were <4.50. The viable count limits for assigning an *Ity* type were chosen on the basis of the 99.9% confidence limits on the geometric means of the progenitor control strains. For those RI strains with lethality between 40 and 60% (that is, BXD-22, -19, -29, -25 and -8), *Ity* types were determined by spleen counts only because this single criterion was used by Plant and Glynn⁶ for mapping the *Ity* locus. The *Ity* types of two RI strains (BXD-28 and BXD-31) were not designated because >60% of the animals died although their geometric mean spleen counts were in the resistant range. The discrepancy between these two parameters in BXD-28 and BXD-31 mice and the intermediate lethality (40–60%) for five BXD RI strains suggest that another gene(s), distinct from *Ity*, is influencing the outcome of infection. This is supported by our observations that the s.c. LD₅₀ of *S. typhimurium* for BXD-28 mice was only 20 (compared with <2 for C57BL/6J mice and 5 × 10⁴ for DBA/2J mice) but the F₁ progeny of BXD-28 and C57BL/6J parents were clearly *Ity*⁺ by spleen counts (log₁₀ geometric mean count for four mice was 2.69). The origin of this hypothetical salmonella sensitivity gene(s) is not clear because progeny from the progenitor strains,

Table 1 *S. typhimurium* susceptibility of BXD recombinant inbred and progenitor strains

Strain	No. dead/total no.	<i>S. typhimurium</i> response $\sin^{-1}\sqrt{\text{no. dead/total no.}}$	$\log_{10}$ geometric mean organisms per spleen (no. of mice)	<i>Ity</i> type	<i>Lsh</i> type*
C57BL/6J	30/30	85 ± 5	6.13 (28)	s	s
BXD-24	11/11	81 ± 8	5.98 (5)	s	s
BXD-12	10/10	81 ± 9	5.46 (4)	s	s
BXD-18	9/9	81 ± 10	5.04 (5)	s	r
BXD-5	8/8	80 ± 10	6.98 (4)	s	s
BXD-20	7/7	79 ± 11	6.33 (3)	s	r
BXD-28	21/22	78 ± 6	4.46 (12)	?	r
BXD-21	6/6	78 ± 12	6.06 (5)	s	s
BXD-31	6/6	78 ± 12	4.28 (7)	?	s
BXD-9	9/10	72 ± 10	7.41 (4)	s	s
BXD-2	10/12	66 ± 8	6.98 (5)	s	s
BXD-14	10/12	66 ± 8	5.80 (5)	s	s
BXD-11	7/9	62 ± 10	5.82 (5)	s	s
BXD-16	7/9	62 ± 10	5.08 (5)	s	s
BXD-22	6/9	55 ± 10	5.10 (5)	s	s
BXD-19	9/14	53 ± 8	6.40 (4)	s	s
BXD-29	7/12	50 ± 8	3.91 (5)	r	s
BXD-25	5/9	48 ± 10	3.37 (5)	r	r
BXD-8	4/8	45 ± 10	2.71 (4)	r	r
BXD-13	3/9	36 ± 10	2.00 (5)	r	r
BXD-15	4/12	36 ± 8	2.28 (5)	r	r
BXD-1	3/12	30 ± 8	2.62 (5)	r	r
BXD-27	2/9	28 ± 10	3.52 (4)	r	r
BXD-23	3/14	27 ± 8	2.26 (5)	r	r
BXD-30	1/12	17 ± 8	4.49 (5)	r	r
DBA/2J	2/25	17 ± 6	3.61 (27)	r	r
BXD-6	0/7	11 ± 11	3.15 (6)	r	r
BXD-32	0/7	11 ± 11	2.20 (5)	r	r

The formula $\phi = \sin^{-1}\sqrt{\text{no. dead/total no.}}$, the arc sin transformation of the raw data, was used for statistical analysis according to ref. 15. The s.e. of ϕ is 828/no. tested. r, Resistant; s, susceptible. Values are means ± s.e.

* The *Lsh* types for BXD-1-29 are taken from ref. 4. The *L. donovani* responses of BXD-31 and BXD-32 were obtained through Dr David Bradley (personal communication).

Table 2 *S. typhimurium* susceptibility of LXB6, 58NXL and BRX58N recombinant inbred and progenitor strains

Strain	No. dead/total no.	<i>S. typhimurium</i> response $\sin^{-1}\sqrt{\text{no. dead/total no.}}$	$\log_{10}$ geometric mean organisms per spleen (no. of mice)	<i>Ity</i> type	<i>Lsh</i> type†
C57BL/6J	30/30	85 ± 5	6.13 (28)	s	s
LXB6-4	11/11	81 ± 9	7.85 (3)	s	s
LXB6-2	3/3	73 ± 17	7.92 (5)	s	s
LXB6-3	4/10	39 ± 9	3.30 (2)	r	r
C57L/J	1/16	14 ± 7	3.48 (4)	r	r
B10.D2(58N)	4/4	76 ± 14	5.90 (6)	s	s
58NXL-3	11/12	74 ± 8	6.69 (4)	s	s
58NXL-4	11/12	74 ± 8	7.08 (6)	s	s
58NXL-2	6/7	68 ± 11	8.00 (4)	s	s
58NXL-1	4/19	42 ± 9	3.59 (3)	r	r
C57L/J	1/16	14 ± 7	3.48 (4)	r	r
58NXL-8	0/13	8 ± 8	3.68 (4)	r	r
BRX58N-13	11/11	81 ± 9	6.51 (5)	s	s, r‡
B10.D2(58N)	4/4	76 ± 14	5.90 (6)	s	s
BRX58N-12	5/8	52 ± 10	6.34 (5)	s	s
BRX58N-7	6/11	48 ± 9	6.83 (7)	s	s
BRX58N-9	4/9	41 ± 9	3.15 (7)	r	r
BRX58N-3	2/11	25 ± 9	3.74 (2)	r	r
C57BR/cdJ	3/20	23 ± 6	4.11 (4)	r	r
BRX58N-10	1/8	21 ± 10	3.50 (5)	r	r
BRX58N-8	0/6	12 ± 12	3.85 (7)	r	r
BRX58N-1	0/7	11 ± 11	2.50 (6)	r	r
BRX58N-11	0/10	9 ± 9	3.16 (4)	r	r

r, Resistant; s, susceptible.

* The statistical analyses are described in Table 1 legend. Values are means ± s.e.

† The *Lsh* types are taken from ref. 4.

‡ Different *Lsh* types were observed in two separate experiments⁴.

that is (C57BL/6J × DBA/2J)F₁ mice, were salmonella resistant (0/4 dead by day 28 when challenged s.c. with 10³ TML) and were able to restrict early *S. typhimurium* net growth in their spleens ($\log_{10}$ geometric mean count for 10 mice was 3.27). As our criteria for *Ity* typing emphasized spleen counts, the influence of this undefined gene(s) on data interpretation was

minimized. Moreover, it is clear that the major gene controlling *S. typhimurium* sensitivity is one closely linked to *Lsh*, since the *Lsh* and *Ity* genotypes were concordant in 21 of the 24 typable BXD RI strains. However, the two genes do not appear to be identical because three strains (BXD-18, -20 and -29) had different responses to *S. typhimurium* and *L. donovani*.

Table 3 *S. typhimurium* susceptibility of LPS-responsive BXH strains and F₁ progeny derived from C57BL/6J and LPS-unresponsive BXH parents

RI strain or F ₁ hybrid	No. dead/total no.	<i>S. typhimurium</i> response $\sin^{-1}\sqrt{\text{no. dead/total no.}}$	$\log_{10}$ geometric mean organisms per spleen (no. of mice)	<i>Ity</i> type of BXH strain	<i>Lsh</i> type of BXH strain†
LPS-responsive					
19	4/13	34 ± 8	ND	r	r
14	0/6	12 ± 12	2.65	r	r
F ₁ from C57BL/6J × LPS-unresponsive BXH					
3	34/35	80 ± 5	ND	s	s
4	8/24	35 ± 6	ND	r	r
7	6/37	24 ± 5	ND	r	r
6	3/33	15 ± 4	ND	r	r
8	0/11	9 ± 8	ND	r	r

The responses of BXH strains to LPS are taken from ref. 11. ND, Not determined; r, resistant; s, susceptible.

* The statistical analyses are described in Table 1 legend. Values are means ± s.e.

† The *Lsh* types are taken from ref. 4.

Genetic studies were performed to verify the *Ity* types of two of these strains. The progeny of crosses between BXD-18 and C57BL/6J mice were tested for salmonella sensitivity and the extent of *S. typhimurium* multiplication in their spleens. We reasoned that if BXD-18 mice are salmonella susceptible as a consequence of *Ity*^s expression, then no gene complementation should be observed in (C57BL/6J × BXD-18)F₁ mice. Thus, our findings that these F₁ mice were sensitive to *S. typhimurium* (4/4 deaths after s.c. infection with 25 organisms) and were unable to control early salmonella growth in their spleens (geometric mean count for nine mice was 6.18) confirm the *Ity*^s genotype of BXD-18 mice. To verify the *Ity*^r genotype of BXD-29 mice, we assessed the spleen counts of individual mice derived from crosses of (C57BL/6J × DBA/2J)F₁ female mice with BXD-29 male mice. Because *Ity*^r is dominant^{2,5,6}, 100% of [(C57BL/6J × DBA/2J) × BXD-29]F₁ mice should be *Ity*^r if BXD-29 animals are *Ity*^r, whereas only 50% should be of this genotype if BXD-29 animals are *Ity*^s. Since all eight of the [(C57BL/6J × DBA/2J) × BXD-29]F₁ mice had spleen counts <10⁵, the *Ity*^r genotype of BXD-29 mice was verified. The salmonella responses of the BXD RI strains therefore suggest that *Lsh* and *Ity* are closely linked, but non-identical, genes. Further studies with LXB6, 58NXL, BRX58N and BXH RI strains confirmed this linkage. No crossovers between *Ity* and *Lsh* or discrepancies between lethality and spleen counts were observed among the 17 strains of LXB6, 58NXL and BRX58N mice tested (Table 2). Experiments with BXH RI strains were complicated by the expression of the defective lipopolysaccharide response gene (*Lps*^d) in some of these strains. As mentioned above, this gene (or one closely linked to it) renders mice susceptible to salmonellae. Moreover, mice homozygous for this allele (*Lps*^d/*Lps*^d genotype) are susceptible to murine typhoid regardless of their *Ity* type⁷. Thus, we expected that all BXH mice of the *Lps*^d/*Lps*^d genotype would be susceptible to infection, and, in fact, all mice of the BXH-2, -3, -4, -6, -7, 8 and -12 strains, which are LPS unresponsive¹¹, died following s.c. infection with approximately 25 *S. typhimurium*. Interestingly, *Lps*^d does not influence the response of mice to leishmaniasis because both *Lsh*^r and *Lsh*^s types occur among these strains⁴. To determine the *Ity* type of some of these LPS-low responder strains, we crossed mice of BXH-3, -4, -6, -7, and -8 with LPS-responsive (*Lps*ⁿ) C57BL/6J mice. As *Lps*^d is recessive and as C57BL/6J mice are *Ity*^r, *Lsh*^r, we were able to use these F₁ mice for *Ity* typing. Such typing was based solely on lethality data because when these experiments were done we did not routinely perform splenic analyses. As indicated in Table 3, the *Ity* and *Lsh* types were concordant in these five hybrid strains. Moreover, the *Ity* and *Lsh* types were also concordant in the two LPS-responsive BXH strains examined (Table 3).

These data provide the first direct evidence for linkage between the chromosome 1 loci, *Lsh* and *Ity*. In only three of 48 *S. typhimurium*-typable strains was there apparent recom-

bination between these markers. Thus, the estimated recombination frequency between *Ity* and *Lsh*, as calculated by the equation of Haldane and Waddington¹², is 0.02 ± 0.01 (s.e. of the estimate). It seems unlikely that the close association of *Ity* and *Lsh* evolved by chance when the 2% recombination frequency is viewed in terms of the entire mouse genome. The advantage of maintaining these loci in close proximity during the evolutionary process is not clear because neither organism appears to be a natural pathogen of feral mice. Nevertheless, the linkage does suggest that the functions of the products of the genes may be related. Since macrophages are critical in the control of both *L. donovani*¹² and *S. typhimurium*¹³ infections, it seems reasonable to presume that these gene products are involved in some phase of the uptake or killing of the organisms by macrophages. One may also speculate, based on such analogous genetic loci as the H-2 and HLA complexes of mice and men, respectively, that man too may have genes which regulate his response to intracellular microbial infections. The concept of a human genetic complex analogous to the murine *Lsh-Ity* complex is not only intriguing but would also have far-reaching clinical implications. Since *Ity*^s mice cannot be protected from murine typhoid by immunization with standard vaccines¹⁴, protection of humans with similar genetic defects may require the development of new vaccines designed specifically to overcome such deficiencies.

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Continuous production of monoclonal rheumatoid factor by EBV-transformed lymphocytes

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Human B lymphocytes are immortalized by Epstein-Barr virus (EBV, ref. 1). The virus can be used to establish lymphoblastoid cell lines that produce and actively secrete specific antibodies². The original method, which we have used for various antigens²⁻⁴ is based on selection of the specific surface antigen receptor-positive lymphocytes from the peripheral blood lymphocytes of a donor who was previously sensitized to the corresponding antigen. Furthermore, by cloning the polyclonal anti-NNP cell line we have produced human monoclonal antibodies for the first time *in vitro*⁵. About 5–20 µg ml⁻¹ stably produced⁵ specific antibody is obtained in the supernatant of the cell lines. This approach can be used for the *in vitro* production of monoclonal human autoimmune antibodies by EBV-immortalized lymphocytes from patients with autoimmune diseases. We demonstrate the continuous production *in vitro* of a monoclonal IgM anti-IgG antibody (rheumatoid factor, r.f.) by a lymphoblastoid cell line established from a patient with rheumatoid arthritis.

Rheumatoid factors that occur in the sera of patients with rheumatoid arthritis are antibodies that react with antigenic determinants on the Fc portion of human IgG. They are heterogeneous with respect to their antigen specificity and can belong to the IgM, IgG or IgA class. In rheumatoid arthritis, rheumatoid factors are usually polyclonal in nature while monoclonal rheumatoid factors have been described in infectious mononucleosis and lymphoproliferative disorders⁶.

We have isolated the lymphocytes from a 15-ml blood sample from a patient (A.N.) with classic rheumatoid arthritis. Standard agglutination tests of the patient's serum using sensitized sheep erythrocytes with rabbit amboceptor (Rose Waaler test) and latex suspension coated with human IgG, gave titres of 1:1024 and 1:80 respectively. Selection and isolation of the antigen receptor-positive cells was carried out by rosetting the lymphocytes⁷ with a 10-fold excess of human IgG coated on erythrocytes and subsequently separating them on a Ficoll-Isopaque gradient (density 1.077)⁵. The rosette-containing pellet was infected with a B958-derived EBV¹. The infected cells were incubated in RPMI 1640 in 20% fetal calf serum (FCS) at 37 °C, 5% CO₂. The emerging cell line contained 0.6% cells that rosetted with human IgG-coated erythrocytes. This very low percentage was apparently due to the fact that in the initial selection stage not only antigen receptor-positive lymphocytes were rosetting with the IgG-coated erythrocytes but also non-related B lymphocytes that had Fc receptors. However, lymphoblastoid cell lines have very weak Fc receptors and usually do not form rosettes with IgG-coated erythrocytes⁸. Therefore we re-selected the emerging line by two subsequent re-rosettings. The outcoming cell line (r.f.-A.N.) is 100% EB nuclear antigen positive⁹. It has a 2-day doubling time and has now been maintained in tissue culture for more than 12 months.

The cells are surface and cytoplasmic IgM λ positive, IgA, IgD and κ negative as shown by staining of live and fixed cells

Table 1 Immunoglobulin classes of r.f.-A.N., and of the r.f.s. produced *in vitro* and *in vivo*

r.f.-A.N. cells and erythrocytes stained	Fluorescence staining for human:						
	Ig	IgM	IgG	IgA	IgD	κ	λ
r.f.-A.N. live cells*	+	+	—	—	—	—	+
r.f.-A.N. fixed cells*	+	+	—	—	—	—	+
Rabbit IgG-OxE exposed to culture supernatant†	+	+	—	—	—	—	+
Goat IgG-OxE exposed to culture supernatant‡	—	—	—	—	—	—	—
DNA OxE exposed to culture supernatant§	—	—	—	—	—	—	—
OxE exposed to culture supernatant	—	—	—	—	—	—	—
Rabbit IgG-OxE exposed to A.N. serum¶	+	+	+	+	—	+	+
OxE exposed to A.N. serum¶	—	—	—	—	—	—	—

* 5 × 10⁵ living cells and a smear of ethanol-acetic acid (95:5)-fixed cells¹⁰ were stained for surface and cytoplasmic immunoglobulins respectively.

† Ox erythrocytes (OxE) coated with sub-agglutinating titre of rabbit anti-ox erythrocytes were incubated with r.f.-A.N. cell supernatant or with A.N. serum for 60 min, washed and stained with Dakopatts FITC-conjugated rabbit anti-human (1), IgG, IgA, IgM, κ and λ, (2) anti-IgM specific for μ chains, (3) anti-IgG specific for γ, Fc fragment, (4) anti-IgA, specific for α chains, (5) anti-K (Bence-Jones), (6) anti-λ (Bence-Jones) and (Behringwerke) rabbit anti-human IgD, specific for δ chains.

‡ Goat IgG was coupled to ox erythrocytes by the CrCl₃ method as detailed in the legend to Table 2. The coating was tested by specific agglutination with rabbit anti-goat IgG (Meloy).

§ Single-stranded DNA was coupled to ox erythrocytes with CrCl₃. The coating was confirmed by specific agglutination of the erythrocytes.

¶ The serum of patient A.N. was absorbed with 2 vol of packed ox erythrocytes before it was used in the assay.

with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human immunoglobulins (Table 1). The nonconcentrated culture supernatant of growing cells (up to 7 × 10⁵ cells ml⁻¹) agglutinated human IgG and rabbit IgG-coated erythrocytes up to 1/128–1/512 dilution; the agglutination was inhibited by 2-mercaptoethanol. A 100-fold concentrated medium of 10⁶ cells growing for 24 h in RPMI with 1.25% FCS had a specific agglutination titre of 1:16,000. By indirect staining of rabbit-IgG-coated erythrocytes previously incubated with the cell line supernatant (Table 1) the specific antibody was identified as an

Table 2 Multi-layer cluster formation by r.f.-A.N. cells

	Cluster formation with rabbit anti-human* IgG (final dilution)					
	None	Ig (10 ⁻⁵)	IgM (10 ⁻³)	IgG (10 ⁻⁴)	IgA (10 ⁻³)	κ λ (10 ⁻³)
Erythrocytes	—	—	—	—	—	—
H-IgG-Ox†	—	+	+	—	—	+
H-IgG-Ox + NaN ₃ ‡	—	—	—	—	—	—
Ox	—	—	—	—	—	—

* 200 washed r.f.-A.N. cells were mixed in a flat-bottomed microplate with 0.02 ml human IgG-coated 10% ox erythrocytes in 0.2 ml RPMI, 20% FCS, with rabbit anti-human immunoglobulins. The rabbit sera used were the unlabelled reagents listed in the legend to Table 1 (Dakopatts). The rabbit antisera were so diluted that they did not agglutinate the IgG-coated erythrocytes. Clusters were examined under a low-power inverted microscope after 4 h incubation at 37 °C.

† IgG coating was performed by mixing 1 vol of saline-washed erythrocytes, 2 vol of 2 mg ml⁻¹ human gammaglobulins (Pentex) in saline and 2 vol of 'aged' 2.5 mM CrCl₃ (ref. 12). The mixture was incubated at room temperature for 5 min followed by three washes with saline.

‡ NaN₃ was used in a final concentration of 0.7 mM.

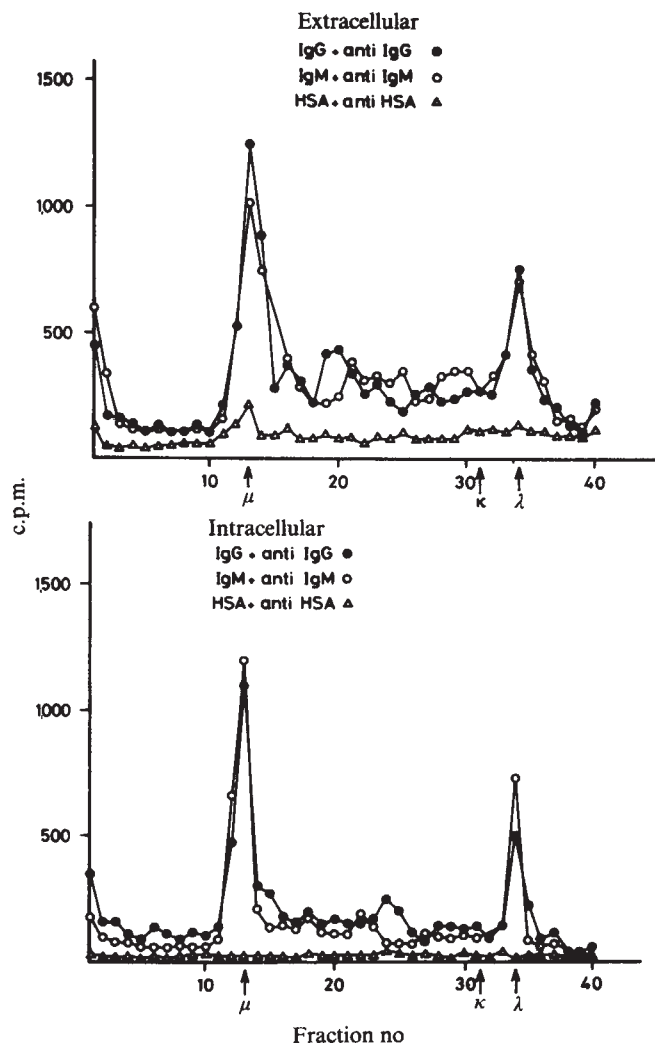


Fig. 1 Gel electrophoresis of immunoglobulins produced by r.f.-A.N. cell line. 12×10^6 r.f.-A.N. cells were incubated in 8 ml of RPMI, amino acid-free medium with 15% dialysed FCS with $40 \mu\text{Ci ml}^{-1}$ of ^3H -labelled amino acid mixture (Amersham) for 24 h. Supernatant was collected, and cells were washed twice and lysed in 2 ml Triton 1%. Both supernatant and cell lysate were dialysed. The supernatant was concentrated 10 times. Aliquots (0.1 ml) of each sample were co-precipitated in a final volume of 0.5 ml with the following antisera and the corresponding antigen in the equivalence zone: rabbit anti-human IgG (Dakopatts) and human IgG; goat anti-HSA (Meloy) and HSA; rabbit anti-human IgM (Dakopatts) and human IgM. The precipitate was washed four times, lysed with 0.1 ml of 1% SDS and SDS-PAGE was performed on 12% acrylamide gels for 4 h (4 mA per tube). The positions of the control markers are indicated by arrows. Cell lysate analyses are shown on the left-hand traces; culture supernatant analyses are shown on the right-hand traces.

IgM, λ . The contrast between the polyclonal anti-IgG that characterizes the patient's serum (A.N.) and the monoclonal type of the anti-IgG of the r.f.-A.N. cell line is evident (Table 1). $65 \pm 10\%$ of the r.f.-A.N. cells form specific rosettes with human or rabbit but not goat IgG-coated erythrocytes or human IgM-coated erythrocytes. The erythrocytes used were: (1) CrCl₃-IgG coated, (2) O, Rh⁺ coated with human IgG anti-D (Ortho), (3) A, Rh⁺ and (4) B, Rh⁺ coated with sub-agglutinating titres of IgM anti-A and anti-B antibody-containing sera.

The high percentage of rosettes is comparable with the specific rosette percentage that characterized the cloned anti-NP lines⁵, which also never reached 100%.

Instead of a plaque assay we developed a method to identify the class of the specific antibody and also the number of cells secreting this antibody, based on the observation of Lubbe *et al.*¹¹. These workers demonstrated the formation of a multi-

layer cluster of antigen-coated erythrocytes around a specific antibody-producing mouse lymphocyte by adding an allogeneic anti-mouse immunoglobulin serum. Table 2 shows that on mixing r.f.-A.N. lymphoblastoid cells with an 100-fold excess of human IgG-coated erythrocytes and diluted rabbit antisera to different classes of human immunoglobulins we detected giant multi-layer clusters. These clusters were formed only in the mixtures containing anti-human total immunoglobulins, IgM or λ chains. No clusters were formed when anti-IgG, -IgA and κ -chains or uncoated erythrocytes were used, and cluster formation was totally inhibited by sodium azide. These results demonstrate that the cells are actively secreting IgM, λ antibodies against human IgG. The significant information this assay provides and the simplicity of its performance make it preferable to the conventional plaque-forming assay.

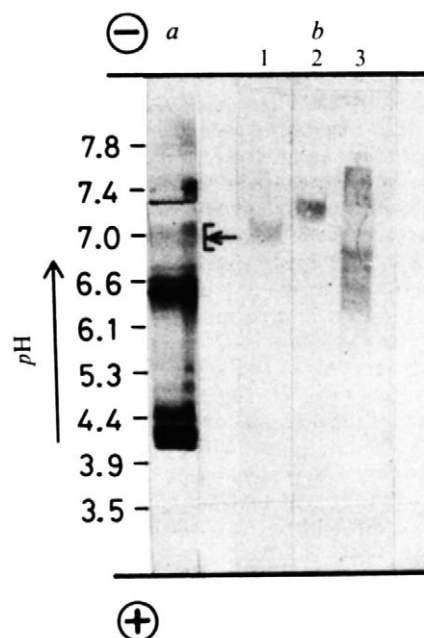


Fig. 2 IEF and immunofixation patterns. *a*, IEF of a concentrated ($\times 1,000$) culture supernatant of the cell line r.f.-A.N. Concentration was performed by precipitation with 50% saturated $(\text{NH}_4)_2\text{SO}_4$ followed by dialysis against H_2O ; the resulting euglobulin was dissolved in 1 ml saline. IgM concentration was $150 \mu\text{g ml}^{-1}$. *b*, Immunofixation patterns obtained with goat anti-human IgM, μ -chain specific serum (Hyland): lane 1, concentrated culture supernatant of the cell line r.f.-A.N.; lane 2, Waldenström's macroglobulinaemia serum (Meloy A 204; 80, 891) diluted 1:75 in saline; lane 3, serum from patient A.N. (undiluted). In all cases 20 μl of the samples soaked into Paratex filter pieces (LKB) were applied on the gel surface. Guiding pH values of the gradient are shown. Agarose IEF was performed in Isogel agarose gel 0.5% (Marine Colloids Division; FMC Corp.) in the presence of a 2% final concentration of Ampholine (LKB) pH 3.5–9.5, supplemented as required by Ampholines with the pH range 2.5–4.0 and 5.0–8.0. The dimensions of the agarose plates were 110×125 mm. The runs were performed in an LKB Multiphor at 5 °C. The current was kept at 10 mA for 10 min, then the Paratex pieces were removed and focusing was continued at 6.25 W constant power (1,000 V limiting) for 1 h. Immunofixation was carried out essentially as described by Pedersen and Axelsen¹⁶.

To characterize the cells and their immunoglobulin product further, they were incubated for 24 h with radioactive amino acid mixture. Both the cell lysate and the culture supernatant were assayed for labelled anti-IgG immunoglobulins by co-precipitation in the presence of human IgG and rabbit anti-human IgG (Fig. 1). The radioactivity is clearly restricted to μ and λ chains. In parallel, co-precipitation of another sample of the same culture supernatant and cell lysate in the presence of human IgM and rabbit anti-human IgM was qualitatively and quantitatively identical to the corresponding co-precipitation with human IgG and rabbit anti-human IgG. This indicates that

all the IgM secreted into the culture medium has anti-IgG activity and that apparently the line is monoclonal.

No radioactive precipitation occurred when goat anti-human serum albumin (HSA) and HSA were added to samples of the cell lysate and the culture supernatant. This is due to the fact that the *in vitro*-produced antibody does not react with goat IgG (see also Table 1).

The clonality of the immunoglobulin product synthesized by the established cell line has been tested by isoelectric focusing (IEF, Fig. 2) in agarose gels, followed by immunofixation. Since the r.f. antibody studied belongs to the IgM class, agarose gels with negligible sieving effects and minimal electroendosmosis were used¹³.

The pattern obtained using the concentrated culture supernatant showed a limited microheterogeneity characteristic for monoclonal IgM antibodies¹³. The pattern is similar to that obtained when a serum from a patient with Waldenström's macroglobulinaemia has been tested in the same run. This provides additional evidence of the monoclonal origin of the r.f. synthesized by the cell line r.f.-A.N.

The presence of a few closely spaced bands in IEF, when the sample containing the intact antibody was analysed (Fig. 2, a, b1) and of a single peak corresponding to the L-chains derived from the same antibody in SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1) are stringent criteria for monoclonality¹⁴. We should mention that the samples tested were crude preparations and that specific procedures to purify the r.f. antibody were not used so as to avoid the selection of a homogeneous population of antibodies.

This is the first time that specific r.f.-producing B cells from rheumatoid arthritis patients have been isolated, immortalized and established as a permanent autoimmune antibody-producing cell line. The availability of such cells can contribute to the understanding of the nature of the cells involved in autoimmune diseases. In addition, this defined product of the cell line is preferable to the unpredictable supply of polyclonal r.f. which is usually used in the radioimmunoassay for detecting immune complexes¹⁵.

Gilliland *et al.*¹⁷ established a cell line with no pre-selection from the synovium of a patient with rheumatoid arthritis that synthesized predominantly IgG, and that had an r.f. activity associated with IgG and IgM. These workers succeeded in establishing such a line after 100 unsuccessful trials from different synovial fluids. Our results suggest an approach to establish autoimmune antibody-producing cell lines that can be extended to other autoantibodies, such as anti-DNA, anti-T.

We have not studied the antigenic specificity of the *in vitro* produced r.f. in relation to the IgG molecule. The *in vitro* produced antibody can be used as an additional tool for understanding the structure and genetics of IgG molecules.

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Relationship between increased aerobic glycolysis and DNA synthesis initiation studied using glycolytic mutant fibroblasts

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Reports from several laboratories have suggested that increased rates of glycolysis play an essential part in the initiation of DNA synthesis. This is based on observations that aerobic glycolysis: (1) occurs at low rate in resting mammalian cells and at very high rate in tumour cells¹⁻³; (2) increases rapidly after DNA synthesis is initiated by addition of serum or purified growth factors^{4,5}, and (3) correlates with the expression of the transformed phenotype⁶. Also, specific inhibitors of aerobic glycolysis prevent the initiation of DNA synthesis⁴. To determine whether the rapid activation of phosphofructokinase—and therefore glycolysis—by purified growth factors⁷ is necessary for the initiation of cell proliferation, we have isolated and studied two classes of glycolytic mutants. The first, isolated from Chinese hamster fibroblasts, has a total block in the glycolytic pathway⁸. The second, from hamster and Fisher rat fibroblasts maintains a permanent high rate of glycolysis. We have found that both classes of mutants retain normal control of DNA synthesis in response to serum. This dissociation indicates that growth-factor-stimulated glycolysis is not involved in the control of initiation of DNA synthesis and cell proliferation.

We used two fibroblast lines requiring serum for growth. The first, 023 which can grow in agarose, is a subclone of Chinese hamster lung fibroblasts (CC139 of ATCC). In spite of a spontaneously-transformed phenotype, its DNA synthesis is dependent on the addition of serum (Fig. 1, inset) or purified growth factors⁹. When CC139 cells are deprived of serum, DNA synthesis is arrested. As with normal cells, DNA synthesis and cell division can be reinitiated by addition of serum. A minimum of 8-10 h is required for the first cells to enter S phase (Fig. 1). The cell population doubles 20 h after stimulation by serum.

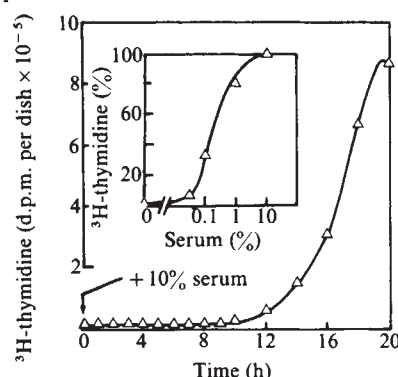


Fig. 1 Re-initiation of DNA synthesis by serum to Chinese hamster fibroblasts. Confluent cultures of Chinese hamster fibroblasts (35-mm dishes) were washed twice with Dulbecco's medium to remove residual serum and then incubated for 29 h in a mixture (1:1) of Dulbecco's and F12 medium (Gibco) deprived of serum. DNA synthesis was re-initiated by addition of 10% fetal calf serum (FCS) and its rate was measured as follows. Cells in duplicate dishes were pulsed for 1 h at 37 °C with 1 ml of culture medium containing 10% serum and ³H-thymidine (5 µCi ml⁻¹). The cells were then washed twice with phosphate-buffered saline and fixed (60 min at 0 °C) with 1 ml of trichloroacetic acid 10% containing 1 mM thymidine. Radioactivity of the acid-precipitable fraction was measured after solubilization in 0.1 M NaOH. The inset shows the rates of DNA synthesis to different concentrations of serum, following 29 h of serum starvation. DNA synthesis was measured after incubation for 24 h with ³H-thymidine (0.5 µCi ml⁻¹). Values are expressed relative to the thymidine incorporation obtained with 10% FCS.

Table 1 Glucose transport and glycolysis in Chinese hamster and Fisher rat fibroblasts and mutant derivatives

	Cell line	Origin	Phenotype	Glucose transport (3-O-MGlc) (nmol mg ⁻¹ min ⁻¹)	Glycolysis (lactate formed) (μmol per mg protein per h)
Chinese hamster fibroblasts	023	CC139 (ATCC)	Wild type	0.110 (100%)	1.4 (100%)
	DS7	023	Glycolysis ⁻ (pgi ⁻)	0.027 (25%)	0.08 (5%)
	GSK3	023	Respiration	0.430 (390%)	3.8 (260%)
Fisher rat fibroblasts	FR3T3	(ref. 10)	Wild type	0.17 (100%)	0.65 (100%)
	GSR16	FR3T3	Respiration ⁻	0.35 (200%)	4.0 (610%)
	FR3T3-Py	FR3T3	Transformed by polyoma virus	0.30 (180%)	2.3 (350%)

All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. 023 is a ouabain-resistant clone derived from the Chinese hamster fibroblasts CC139 (ATCC). DS7 and GSK3 were derived from 023 cells after mutagenesis with ethylmethane sulphonate as described for DS7⁸. The selection of the respiration-deficient mutants, GSK3 and GSR16 has been described elsewhere¹³. Cells were planted in 35-mm dishes, cultured to near confluency in 2–3 days, and assayed for 3-O-methyl D-glucose. For 3-O-MGlc transport studies, cells were precharged with 50 mM 3-O-MGlc before transport and assayed with 50 μM 3-O-MGlc. Rates of transport are the average of duplicate determinations with time points of 15, 30 and 60 s. Rates of glycolysis were calculated from the linear kinetic (lactic acid produced into the medium) obtained with time points of 30, 60 and 90 min.

Therefore, as with normal cells, growth-factor deprivation reversibly arrests these Chinese hamster cells in the G₀/G₁ phase of the cell cycle. The second cell line was isolated by Seif and Cuzin¹⁰ from Fisher rat embryo fibroblasts. This diploid line, FR3T3, is anchorage-dependent, non-tumorigenic and dependent on serum for growth.

To analyse the relationship between increased glycolysis and the initiation of DNA synthesis, we searched for a class of mutants in which glycolysis is suppressed and another in which it is permanently derepressed. We used tritium 'suicide' to select these mutants. From hamster fibroblasts treated with ³H-labelled 2-deoxyglucose we selected DS7, a mutant lacking

phosphoglucose isomerase activity. (Details of the selection are given elsewhere⁸.) By a similar method, but using [6-³H]-D-glucose, we isolated GSK3 from hamster cells and GSR16 from rat fibroblasts. Some of the clones surviving ³H-glucose 'suicide' had a specific defect in the oxidation of glucose by the Krebs cycle. This was due to a block in respiration that resulted in a decrease in oxygen uptake. One consequence of this mutation is a decrease in ATP derived from oxidative metabolism; this in turn triggers a permanent activation of glycolysis to compensate for the deficit in energy.

Table 1 summarizes rates of glucose transport and glycolysis in the two cell lines and their mutant derivatives. The rate of glycolysis is decreased 20-fold in DS7, whereas it is increased six-fold in GSR16 (compared with the corresponding parental lines). Interestingly, in conjunction with a block in glycolysis, DS7 has low glucose transport activity, whereas derepression of glycolysis in GSK3 or GSR16 is accompanied by increased glucose transport. The biochemical basis for this co-regulation will be discussed elsewhere¹¹. It is also interesting that the respiration-deficient mutation introduced into FR3T3 activates glycolysis and glucose transport to values higher than those observed in the highly transformed polyoma virus derivative, FR3T3-Py (Table 1).

Figure 2 shows that re-initiation of G₀/G₁-arrested wild type 023 cells by serum rapidly stimulates (sixfold) glycolysis. In contrast, as would be expected, serum cannot stimulate glycolysis in the deficient mutant DS7. However, re-initiation of its DNA synthesis is not prevented by the glycolytic defect. Even in conditions of serum starvation, GSK3 sustains levels of glycolytic activity 10 times higher than those in wild-type arrested cells, and even higher than in serum-stimulated 023 cells. Although serum starvation fails to reduce rates of glycolysis and glucose transport to low levels, DNA synthesis is arrested in GSK3 cells (Fig. 2).

Results obtained with the untransformed cell line FR3T3 and its derivatives are shown Fig. 3. As with GSK3, when the respiration-deficient mutant GSR16 is starved of serum for 48 h, its rate of glycolysis is not reduced to low levels: indeed it is slightly higher than in polyoma-virus transformed FR3T3 and 10 times higher than in normal FR3T3. However, Fig. 3 shows that in contrast to FR3T3-Py in which DNA synthesis cannot be arrested, GSR16, like the parent FR3T3 line, has normal control of DNA synthesis and cell division.

Thrash and Cunningham¹² have demonstrated a dissociation of increased transport from initiation of fibroblast proliferation. Our studies extend this dissociation to aerobic glycolysis and support their report because DS7 and GSK3 have a 15-fold difference in glucose transport (Table 1), yet they both show normal control of DNA synthesis.

Zielke *et al.*¹³ have reported that human fibroblasts can undergo one to two cell divisions in the absence of glucose, provided that the anabolic function of glucose required for nucleic acid synthesis is substituted by the addition of purines

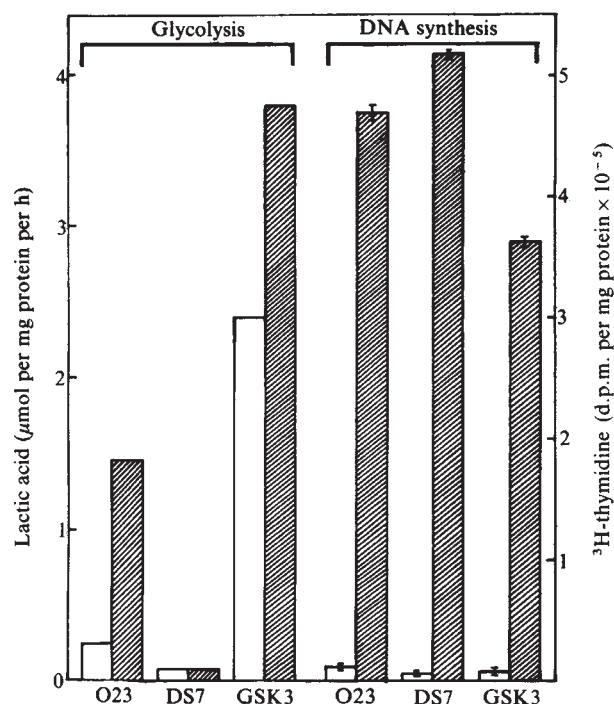


Fig. 2 Rates of glycolysis and DNA synthesis in serum-starved and serum-refed Chinese hamster fibroblasts. Wild type (023) and mutant (DS7 and GSK3) cells were grown for 2 d in media containing 10% FCS (10⁶ cells per 35-mm culture dish). The cells were then washed (twice to remove residual serum) and incubated for 29 h in the mixture (1:1) of Dulbecco's and F12 medium. At the end of the starvation period, media were renewed and half of the cells were supplemented with 10% FCS (hatched bars). Rates of glycolysis were measured as the amount of lactic acid produced during the first hour after starvation (see legend to Table 1). Rates of DNA synthesis were determined by the incorporation of thymidine into acid-precipitable material after 24 h of incubation in the presence (hatched columns) or absence (open columns) of 10% serum and ³H-thymidine (0.2 μCi ml⁻¹).

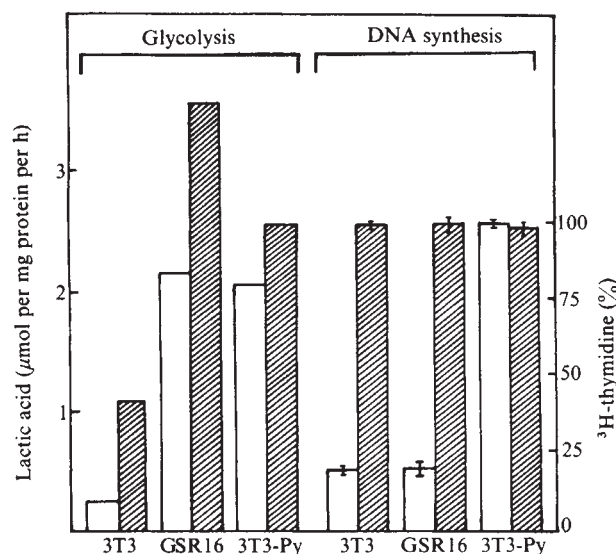


Fig. 3 Rates of glycolysis and DNA synthesis in serum-starved and serum-refed Fisher rat fibroblasts. FR3T3, GSR16 and FR3T3-polyoma transformed cells (10^5 per 35-mm dish) were grown for 2 d in 10% FCS and then starved of serum for 48 h in the mixture (1:1) of Dulbecco's and F12 medium. Then half the cells were supplemented with 10% FCS. Rates of glycolysis and DNA synthesis were measured as for Fig. 2. Open columns, cells starved of serum; hatched columns, cells with 10% serum added.

and pyrimidines to the medium. Therefore, one may ask whether a possible role of glucose metabolism in controlling DNA synthesis or cell proliferation *in vivo* has been masked in *in vitro* studies, in which a rich medium (DMEM/F12) containing nucleic acid precursors was used. There are two lines of evidence against this possibility. (1) The results shown in Fig. 2 for 023 and DS7 were also observed when the cells were serum-starved and serum-stimulated in Dulbecco's medium alone (not containing the nucleic acid precursors of the F12 medium). In this situation the anabolic function of glucose is required and the pentose-phosphate shunt, still functional in DS7, facilitates the re-initiation of DNA synthesis. (2) We have shown that the glycolytic block in DS7 cells does not prevent their capacity to form tumours⁸ and therefore to proliferate *in vivo*.

In conclusion, the properties of GSK3 and GSR16 indicate that the loss of growth regulation of transformed or tumour cells is not linked to their high glucose metabolism. Also it seems clear from the study of the glycolysis-deficient mutant DS7 that growth factor-activated glycolysis or glycolysis *per se*, is not necessary for the initiation of DNA synthesis and cell division.

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Single Na⁺ channel currents observed in cultured rat muscle cells

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The voltage- and time-dependent conductance of membrane Na⁺ channels is responsible for the propagation of action potentials in nerve and muscle cells. In voltage-step-clamp experiments on neurone preparations containing 10^4 - 10^7 Na⁺ channels the membrane conductance shows smooth variations in time, but analysis of fluctuations^{1,2} and other evidence³ suggest that the underlying single-channel conductance changes are stochastic, rapid transitions between 'closed' and 'open' states as seen in other channel types. We report here the first observations of currents through individual Na⁺ channels under physiological conditions using an improved version of the extracellular patch-clamp technique⁴⁻⁶ on cultured rat muscle cells. Our observations support earlier inferences about channel gating and show a single-channel conductance of approximately 18 pS.

Our cell preparations were myoballs^{7,8} prepared from 19-20-day old rat embryos. After fusion to form myotubes the cells were treated for 2 days in 10^{-6} M colchicine, which caused them to assume a spherical shape, and then returned to the normal growth medium (Dulbecco's minimal essential medium with 10% fetal calf serum) for 2-5 days before use. As excitable cells, myoballs were advantageous for several reasons. First, Na⁺ channels in frog muscle show longer activation time constants than those in frog nerve, and we observe slower activation in myoballs than in mammalian nerve⁹. The long time constants reduce the time-resolution requirements of the recording system. Second, the absence of connective tissue in the cell culture allows direct access to the sarcolemma without enzyme treatment⁵. And third, the spherical shape of the cells allows the use of a conventional, two-microelectrode voltage clamp for measurement of the whole-cell currents. In contrast to the recent recordings from K⁺ channels in squid axons by Conti and Neher¹⁰, we were also able to use physiological ionic solutions for our experiments. We did, however, cool the cells to room temperature (19-22 °C) to slow the channel kinetics.

The recording method (Fig. 1) represents an order-of-magnitude improvement in resolution over previous results from the patch recording technique. The basis of the improvement is an increase from 10^8 to 10^{10} Ω of the seal resistance that electrically isolates a small patch of membrane for current measurement⁶. The higher resistance introduces less thermal noise (which in the past was the predominant noise source) allowing a wider recording bandwidth to be used. It also allows voltage steps to be applied to the patch without the necessity of voltage-clamping the entire cell with microelectrodes. A seal is formed by applying gentle suction in the pipette which is pressed against the cell membrane. In two experiments we estimated the membrane patch area to be 2 and 10 μm² from transfer capacitance measurements on cells that were also voltage-clamped with microelectrodes. This area is large in view of the 0.3-0.5 μm diameter of the opening in the pipette tip, and suggests that part of the membrane is drawn into the pipette, forming an omega shape (Fig. 1).

The size of the peak Na⁺ currents recorded with the micro-electrode clamp in these cells varied from essentially zero to about 1 mA per cm² of surface area; this latter current density corresponds to about 10 channels per μm² surface area. Capacitance measurements⁷ suggest that the total membrane area is much larger than the surface area, as would be expected from transverse tubules or extensive membrane infolding. The

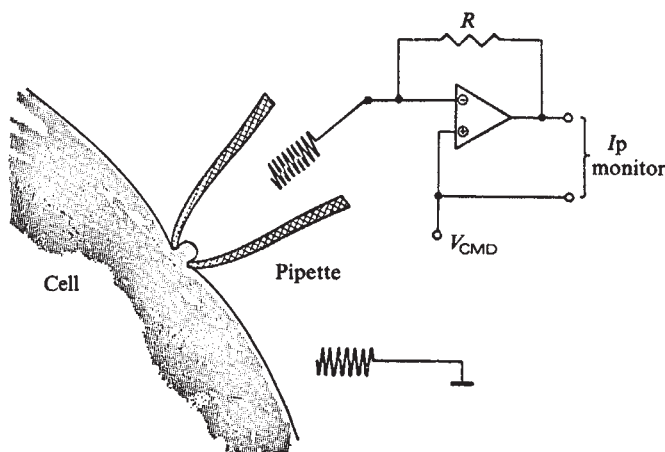


Fig. 1 Recording system. Details of pipette fabrication and use are given in ref. 6. Briefly, a freshly fire-polished glass pipette with inner opening diameter $\sim 0.3\text{--}0.5\ \mu\text{m}$ is filled with modified Ringer solution and pressed against a myoball (cell diameter $30\text{--}80\ \mu\text{m}$). Suction ($5\text{--}50\ \text{cm H}_2\text{O}$) is applied to the inside of the pipette for $5\text{--}30\ \text{s}$ until the sealing resistance, measured as the current response to small voltage pulses at V_{CMD} , suddenly increases to $> 1\ \text{G}\Omega$. Ag-AgCl electrodes are used in the pipette and for the reference electrode in the bath. The patch clamp circuitry resembles that described previously⁵, but was modified to exploit the high seal resistance. The current measuring resistance R was increased to $10\ \text{G}\Omega$, and frequency-response corrections were applied in a later amplifier stage to maintain flat response in the current monitor signal to $7\ \text{kHz}$. The operational amplifier is a Burr-Brown 3523J. As the input shunt capacitance ($\approx 3\ \text{pF}$) causes the voltage noise of the amplifier to predominate at high frequencies, we coat the pipettes with wax or Sylgard to reduce the wall capacitance. The potential inside the pipette is controlled by V_{CMD} ; this also provides very good control of the potential of the membrane patch since the pipette access resistance is $< 10\ \text{M}\Omega$ and the patch currents cause negligible changes in the resting potential of the cell. The bathing Ringer solution contained (in mM): $140\ \text{NaCl}$, $1.4\ \text{KCl}$, $2.0\ \text{MgCl}_2$, $1\ \text{CaCl}_2$ and $20\ \text{HEPES}$ at $\text{pH}\ 7.4$. The pipette solution was the same with $0.2\text{--}1\ \mu\text{g ml}^{-1}\ \alpha\text{-bungarotoxin}$ added to block acetylcholine receptors. Both solutions were filtered ($0.2\ \mu\text{m}$ pore size) immediately before use. Occasionally $5\ \text{mM TEA Cl}$ (replacing NaCl) was also present to block K^+ channels; this had no obvious effect on the patch current recordings.

small number of channels in a patch—at most 3–5 channels were simultaneously active—is consistent with this. Also, voltage clamp of the cells sometimes showed large, tetrodotoxin (TTX)-sensitive regenerative responses at small depolarizations, characteristic of a large membrane component under poor voltage-clamp control. No responses of this kind were seen in the patch clamp recordings, however.

Figure 2c shows a series of current records from a membrane patch, obtained by successive depolarizations to $V = 10\ \text{mV}$, where V is the displacement from the resting potential. Discrete current pulses of varying duration appear after the start of the depolarizations; inactivation of the currents appears as a reduction in the frequency of appearance later in the depolarizations. In roughly half of the records no channel events were seen; in two records of the 300 taken in this run two overlapping events were seen.

Conditions known to reduce the Na^+ current also affect these current pulses. When two-thirds of the Na^+ in the pipette was replaced with the impermeant substitute tetramethylammonium ion (TMA), the discrete currents were reduced in size (Fig. 2d). In other experiments the sensitivity to block by TTX was tested statistically. For these we used two culture dishes of cells 5 days after colchicine treatment because at this later stage the channel density seems to be more uniform¹¹, as reflected by the fraction of patches that contain channels. In each dish four recordings were made, three with Ringer solution in the pipette and one with $1\ \mu\text{M TTX}$ added. Channel activity was present in all six

patches in the absence of TTX, and not present in TTX, showing that TTX blocks at least some of the channels ($P < 0.05$, Fisher test).

Assuming that the Na^+ -channels in these cells are identical and function independently, one expects that the sum of many current records from a few channels should show the same properties as the sodium current measured in a conventional voltage-clamp from thousands of channels. Such an average time course derived from 300 depolarizations of the membrane patch is shown in Fig. 2b. It shows the typical kinetic behaviour¹² with a rapid ($\tau_m \approx 0.8\ \text{ms}$) activation followed by a slower ($\tau_h \approx 2.6\ \text{ms}$) inactivation process.

Averages and representative single records are also shown in Fig. 3 for variously sized depolarizations. With increasing depolarization the peak inward current first increases, as the probability that channels are open rises, and then decreases as the single-channel currents become small near the reversal potential. The mean channel open time, τ , shows the qualitative behaviour expected from Hodgkin-Huxley kinetics¹² where $\tau = 1/(3\beta_m + \beta_h)$. At small depolarizations τ increases with depolarization as the closing rate constant β_m decreases. At larger depolarizations the increasing inactivation rate β_h shortens τ . There is, however, a quantitative disagreement, with τ not differing significantly from τ_m in the data of Fig. 2 and in two other experiments at $V = 10$ and $20\ \text{mV}$. At such small depolarizations, where activation is small, τ would be near $\tau_m/3$ in the Hodgkin-Huxley theory. The channel lifetimes are consistent, on the other hand, with the kinetic theory of Armstrong and Gilly¹³ in which the final opening step of the channel is rate limiting in channel activation.

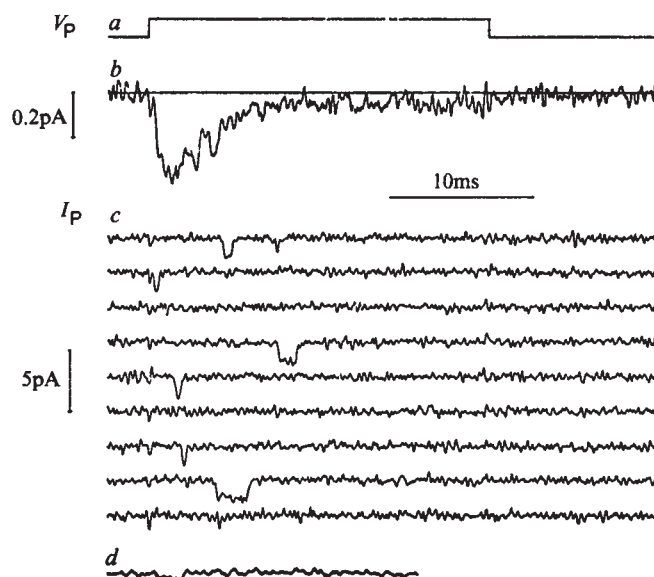


Fig. 2 Single-channel currents during $10\ \text{mV}$ depolarizations. *a*, Imposed patch membrane potential. The patch was held hyperpolarized by $30\ \text{mV}$; $40\ \text{mV}$ depolarizing pulses were given at 1-s intervals. *b*, Average of a set of 300 current records elicited by these pulses. *c*, Nine successive individual records from this set. The mean channel current was $-1.6\ \text{pA}$, and lifetime was $0.7\ \text{ms}$. *d*, Example of a current record obtained in another experiment where two-thirds of the Na^+ in the pipette was replaced with tetramethylammonium ion. The record was plotted on the same scale as *c* but additional filtering was used to reduce the noise level. Leakage and capacitance currents were subtracted in each case using means of responses to symmetrical hyperpolarizing pulses; to remove a residual capacitance artefact in *b* and *c* a mean time course was computed from records in which no channel activity was visible, and this mean was subtracted from individual records. Filtering of the data resulted in effective system risetimes of 300 , 180 and $300\ \mu\text{s}$ in *b*, *c*, and *d*, respectively. The patch apparently contained 2–3 active channels, as estimated from responses to larger depolarizations. The solution in the pipette contained $5\ \text{mM TEA}$. Cell 3.1; temperature, 18°C .

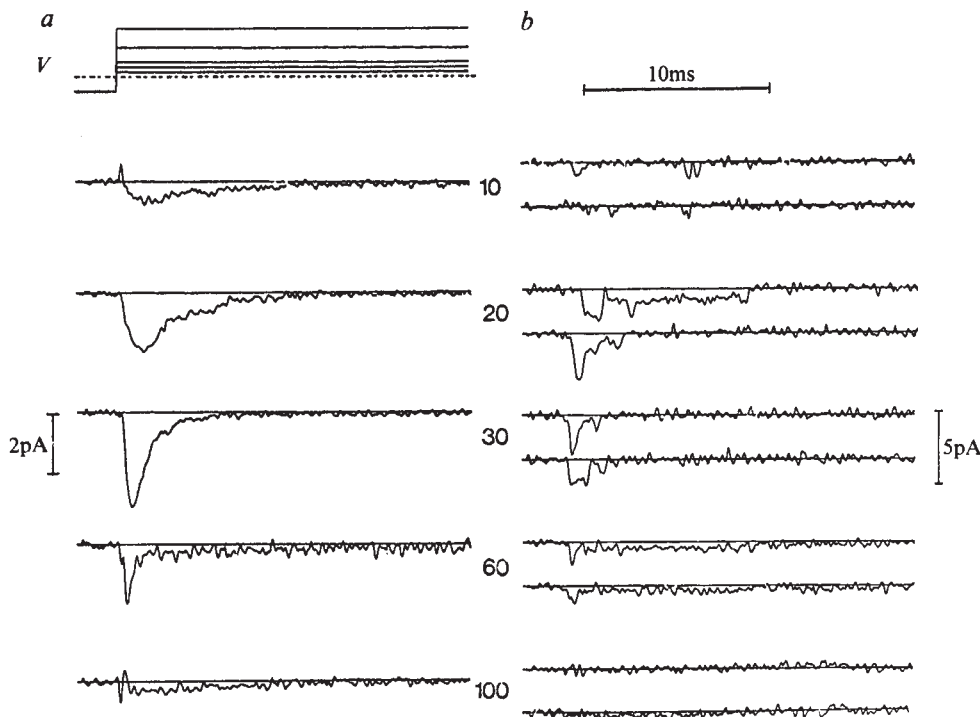


Fig. 3 Patch currents obtained with depolarizations to the various potentials V shown. *a*, Means calculated from 24–104 current records; *b*, representative individual records at each potential. Overlapping currents from 2–3 channels can be recognized at $V=20$ and 30 mV. Note the decrease in current size near the reversal potential (approximately $V=100$ mV). Leakage and capacitance currents were subtracted with averages from symmetrical hyperpolarizations. Recording system risetime was $220\ \mu\text{s}$. Cell 7.1, 3 days after colchicine treatment. Temperature, 22°C .

Estimates of the cell resting potentials allow us to express values of V in terms of absolute membrane potential. Impalements with microelectrodes showed maximum resting potentials in the range -60 to -70 mV. h_∞ curves from averaged patch currents showed $h_\infty=0.5$ in the range $V=0$ to -15 mV whereas measurements with the microelectrode voltage clamp showed corresponding absolute potentials of -80 to -95 mV. We conclude therefore that the resting potentials were in the range -60 to -80 mV. This places the apparent reversal potential $V_{\text{Na}} \approx 100$ mV in Fig. 3 in the absolute membrane potential range of $+20$ to $+40$ mV.

The size of the single-channel currents corresponds to a single-channel conductance $\gamma = 18$ pS. This value is at first sight surprisingly large, as γ estimates for frog nerve Na^+ channels, based on current fluctuation analysis, are in the range 6 – 8 pS (refs 1, 2). These values become twice as large, however, when they are corrected to the temperature and higher sodium concentration used in our experiments, assuming $^{14}Q_{10} = 1.3$ and a linear concentration dependence of conductance.

The extracellular patch clamp as used in this work promises to be a powerful electrophysiological technique for tissue-cultured cells. The ability to clamp and change the potential in small patches of membrane now allows voltage-clamp studies to be performed on cells that are too small or have unsuitable geometries for microelectrode work.

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Localization of [^3H]-2-deoxyglucose in single molluscan neurones

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The glucose analogue 2-deoxyglucose (2-DG) can be used quantitatively to measure metabolic activity¹ and is widely used qualitatively for mapping functional activity in the brain^{2–4}. The resolution (meaning the full width at half maximum of the grain density distribution around a line source) of the technique using [^{14}C]-2-DG and X-ray film is limited to about $100\ \mu\text{m}^5$. Attempts have been made to improve the resolution using [^3H]-2-DG (ref. 6) and cellular resolution has been achieved in the goldfish retina⁷ and with cultured mouse neurones⁸. An anatomical technique for mapping the metabolic activity of individual neurones would be useful for studying invertebrate central nervous systems, which are relatively simple and stereotyped compared to vertebrate brains. The [^3H]-2-DG technique was applied to an invertebrate in a study of the *Drosophila* visual system⁹, though without cellular resolution. We present here modifications of the [^3H]-2-DG technique to demonstrate localization of 2-DG in single neurones of *Limax maximus*, a gastropod mollusc, with a resolution of less than $1\ \mu\text{m}$.

Glucose and 2-DG compete both for transport into and out of a cell and for phosphorylation by hexokinase inside the cell. Once 2-DG is phosphorylated to 2-deoxyglucose-6-phosphate (2-DG-6-P), it is not metabolized further and accumulates in the cell. In the conventional [^{14}C]-2-DG technique¹, the tissue is frozen, sectioned on a cryostat, picked up on a warm coverslip

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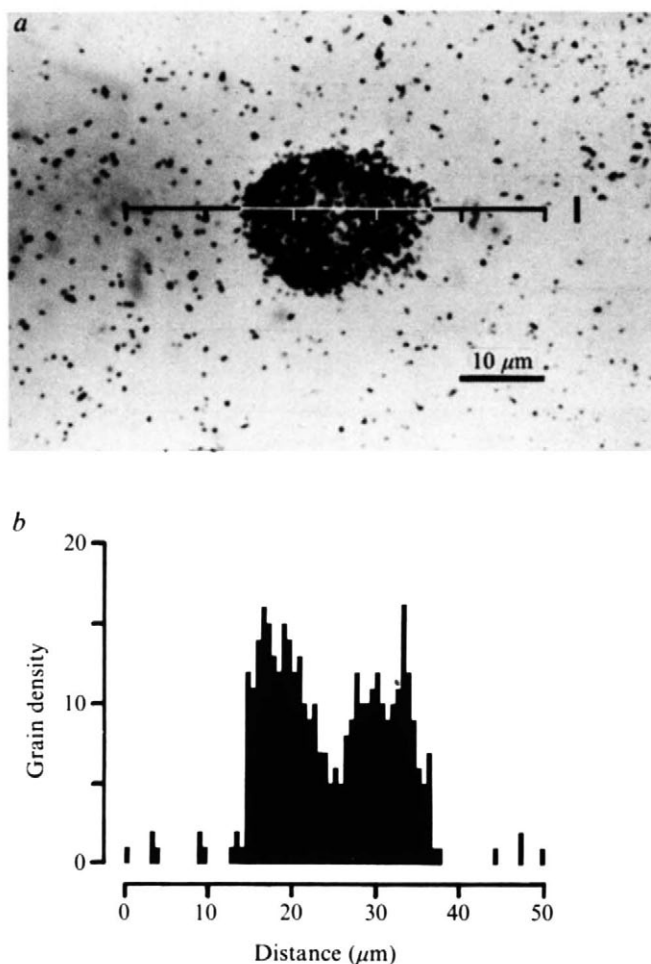


Fig. 1 Measurement of silver grain density over a single cell body in a buccal ganglion. *a*, Light-microscopic autoradiograph from a preparation incubated in [^3H]-2-DG for 45 min and freeze-substituted with acetone at -70°C . During the incubation, a neurone in the buccal ganglion was stimulated with an intracellular electrode to elicit action potentials at the rate of 2 s^{-1} . Several cells in the buccal ganglion were labelled. Scale bar, $10\text{ }\mu\text{m}$. *b*, A reticle with 100 lines was superposed over the cell at high magnification and the silver grains were counted by focussing through the emulsion of the autoradiograph. The number of silver grains between reticle lines (in a rectangle $0.63 \times 4.6\text{ }\mu\text{m}$) as shown to the right of the cell) is plotted as a function of distance along the line drawn through the cell body in *a*. The drop in density of silver grains over the centre of the cell occurs over the nucleus. The density of silver grains over the cytoplasm is $4.1\text{ }\mu\text{m}^{-2}$, which is 40 times the average background density over the tissue surrounding the cell and 290 times the background density in the emulsion.

and dried on a hot plate, during which the 2-DG and 2-DG-6-P can freely diffuse and interfere with the localization of the label within cells. One way to prevent diffusion is to replace the water in the cell with a solvent in which 2-DG and 2-DG-6-P are insoluble and to exclude water from the tissue in all subsequent steps. We have localized [^3H]-2-DG using two methods: in the first, the tissue was frozen and freeze-substituted¹⁰ with acetone at -70°C , and in the second, the tissue was dehydrated in dry acetone at 4°C .

The buccal ganglion of *L. maximus* innervates the animal's feeding musculature and contains several large identifiable neurones as well as several hundred smaller cells^{11,12}. The stereotyped structure of the ganglion, its small size (about 1 mm in diameter) and its accessibility to electrophysiological techniques make it suitable for studying the uptake of 2-DG by single neurones. In each experiment, a pair of buccal ganglia was

dissected and placed in saline modified by lowering Ca^{2+} to 0.07 times its normal concentration and raising Mg^{2+} by five times to reduce transmission at chemical synapses. (The composition of the solution was 56 mM Na^+ , 4.2 mM K^+ , 0.5 mM Ca^{2+} , 23 mM Mg^{2+} , 104 mM Cl^- , 0.2 mM H_2PO_4^- , 2.5 mM HCO_3^- and 5 mM dextrose.) Approximately $100\text{ }\mu\text{Ci ml}^{-1}$ of 2-[1,2- ^3H]-deoxy-D-glucose (New England Nuclear NET-549, specific activity 40 Ci mM^{-1}) was added to the bath and the ganglia were incubated for 1 h or less, depending on the experiment. Afterwards, the ganglia were rinsed in five washes of fresh saline over 30 min. For freeze-substitution, the ganglia were then rapidly frozen in Freon 22 cooled to -160°C with liquid nitrogen, placed in a vial containing 20 ml dry acetone and 1 g of Al_2O_3

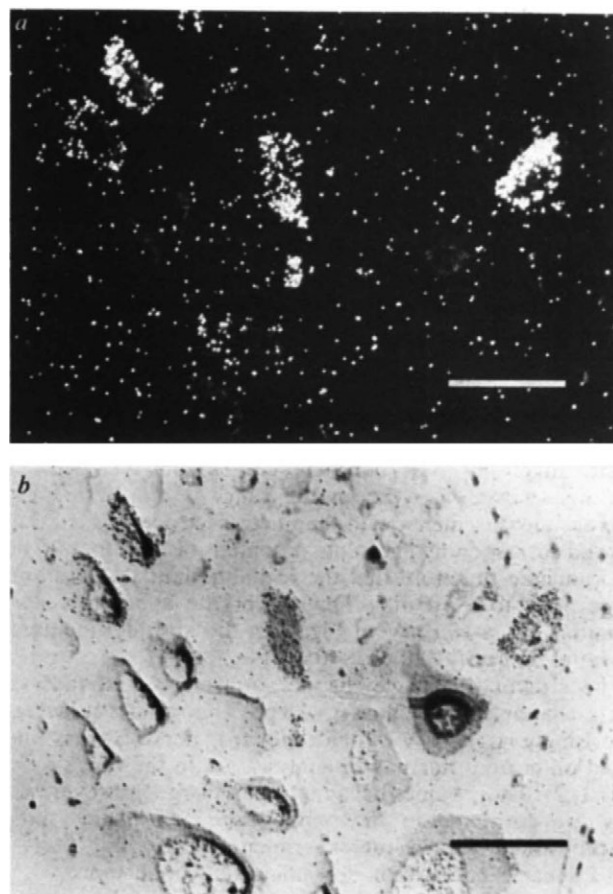


Fig. 2 Light-microscopic autoradiograph of several labelled cell bodies in a buccal ganglion. The ganglion was incubated in [^3H]-2-DG for 20 min and dehydrated in dry acetone at 4°C . During the incubation a neurone was stimulated with an intracellular electrode to elicit action potentials at the rate of 2 s^{-1} . The sections were lightly stained with methylene blue. *a*, Five labelled cells (dark-field illumination). The cell bodies could be traced in serial sections through the ganglion. The nuclei of the two labelled cells on the far left and the cell on the far right were not labelled above background. The average density of silver grains over the labelled cells was $0.1\text{ }\mu\text{m}^{-2}$ – $0.5\text{ }\mu\text{m}^{-2}$, which was approximately 10–50 times the background density over surrounding tissue. *b*, The same section in *a*, photographed using Nomarski differential interference contrast optics. Scale bar, $40\text{ }\mu\text{m}$.

(Brockman grade 1), and kept at -70°C for 48 h. For acetone dehydration, the ganglia were placed in an identically prepared vial and kept at 4°C overnight. No fixatives were used. (Fixation with glutaraldehyde^{6,7} results in a loss of over 90% of the radioactivity from the tissue^{7,8}.) After either freeze-substitution or dehydration, the tissue was gradually warmed to room temperature and embedded in Araldite to which 1% by weight Dow-Corning silicone fluid¹³ had been added. The embedded

tissue was then dry-sectioned at 2–4 μm on an ultramicrotome and placed on a slide coated with a thin layer of anhydrous glycerol. The glycerol was evaporated by setting the slide on a hot plate at 110 °C. The slides were then dipped in Kodak NTB-2 nuclear emulsion, dried, exposed at 4 °C for 2–4 weeks, developed in Kodak D-19 at 16 °C for 6 min, washed and stained with either 1% methylene blue or 1% toluidine blue.

Scintillation counting was used to determine the uptake of [^3H]-2-DG by ganglia during incubation and the loss of label during subsequent processing. The amount of radioactivity left in the acetone following either freeze-substitution or dehydration was less than 0.5% of the total radioactivity remaining in the ganglion. The loss of label during autoradiography was not quantitatively assessed, but it was probably low because spreading of label around the edges of the section or around discretely labelled structures within the section was never seen.

Autoradiographs of buccal ganglia incubated in [^3H]-2-DG revealed labelled individual neurones in all preparations. No cells were labelled in control ganglia incubated without [^3H]-2-DG, thus ruling out positive chemography. In seven heavily labelled cells from three preparations, the density of silver grains over cell bodies was between 0.1 μm^{-2} and 4.5 μm^{-2} , which was 10–50 times the mean background density over adjacent tissue. The glucose utilization is therefore 10–50 times greater within some cells than without. The localization of [^3H]-2-DG in a single heavily labelled buccal neurone is shown in Fig. 1a. The density of silver grains drops sharply at the boundary of the cell body, with the transition from maximum grain density to background occurring in less than 1 μm (Fig. 1b). The resolution of the technique appears to be limited by the size of a developed silver grain, which has a diameter of 0.5 μm . Whenever the stained cell membrane of a labelled cell is visible, the grain density drops abruptly at the cell membrane. In some labelled cells, such as those shown in Fig. 2, the grain density also drops abruptly at the nuclear membrane, with much lower labelling over the cell nucleus than the cytoplasm. In addition to labelled cell bodies, labelled structures could also be detected in the neuropil.

The high resolution obtained with this technique is probably the result of removing water from the tissue. During freeze-substitution, the maximum concentration of water at the interface between ice and acetone is 4% at –70 °C (ref. 14). Thus, molecules insoluble in acetone diffuse very little from the site at which they were immobilized during freezing. For example, freeze-substitution has been used to localize Ca^{2+} in subcellular compartments¹⁵. More diffusion of 2-DG and 2-DG-6-P occurs within the cell with acetone dehydration at 4 °C than with freeze-substitution at –70 °C. However, the labelling of single cell bodies using acetone dehydration had the same resolution as with freeze-substitution. We can therefore conclude that both freeze-substitution and acetone dehydration are effective in localizing [^3H]-2-DG within single neurones.

The [^3H]-2-DG technique presented here can be used to measure the qualitative change in the distribution of label under different conditions. Preliminary experiments indicate that the labelling of single neurones demonstrated here is correlated with spontaneous and stimulated electrical activity in the ganglion¹⁶. The technique could be useful in studying networks of neurones underlying behaviour in the isolated central nervous system¹⁷. In particular, the participation of small neurones, which are difficult to study electrophysiologically, can be monitored as easily as large neurones. It may also be possible to measure the metabolic activity of axon terminals in the neuropil. As the resolution reported here is close to the limiting resolution of light microscopy, further improvement will require the use of electron microscopy.

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Repeated tricyclics induce a progressive dopamine autoreceptor subsensitivity independent of daily drug treatment

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Dopamine (DA) has largely been ignored in considering possible mechanisms underlying the therapeutic effects of tricyclic anti-depressants (TCA)^{1–3}. Most previous work done has focused on the ability of some TCAs to block the *in vitro* re-uptake of DA¹—an effect which unfortunately requires very high doses. Recently, however, Serra *et al.*³ proposed that TCAs may exert their therapeutic effects by inducing a subsensitivity of presynaptic receptors located on the dendrites and soma of DA neurones (DA autoreceptors). This hypothesis was directly tested by examining the influence of TCAs on the demonstrated ability of the DA agonist, apomorphine, to depress selectively the spontaneous activity of single DA cells. We now report that repeated administration of both typical and atypical TCAs induces a progressive subsensitivity of DA autoreceptors, and that this gradual augmentation of DA autoreceptor subsensitivity depends on the passage of time rather than daily TCA administration. The latter finding suggests that daily drug administration may not be therapeutically necessary.

The 53 male albino rats (200–250 g; Zivic Miller) used in these studies were housed two per cage with free access to food and water and maintained on a 12 h light/dark cycle. Animals were handled and weighed daily for 2 weeks before experiments began. All experiments were done in double-blind conditions. After a period of drug treatment (specified below) the animals were anaesthetized with chloral hydrate [400 mg per kg, intraperitoneally (i.p.)], mounted in a stereotaxic apparatus and the spontaneous discharge rates of single DA neurones located within the zona compacta of the substantia nigra (SN; coordinates: anterior 1,300–2,400 μm , lateral 1,300–2,400 μm [ref. 4]) were monitored as previously described^{5,6}. Briefly, extracellular electrical activity was recorded through glass micropipettes filled with 2 M NaCl which was saturated with fast green dye (tip size 1–3 μm , *in vitro* d.c. impedance of 4–12 M Ω). Neuronal signals were fed into a high input-impedance differential amplifier (bandpass filters: 100 Hz and 3 kHz) whose output was led into a window discriminator, a signal integrator and digital frequency meter. The femoral vein was catheterized for the intravenous (i.v.) administration of all pharmacological agents. All drug doses are based on the weight of the salt. At the termination of each experiment the recording

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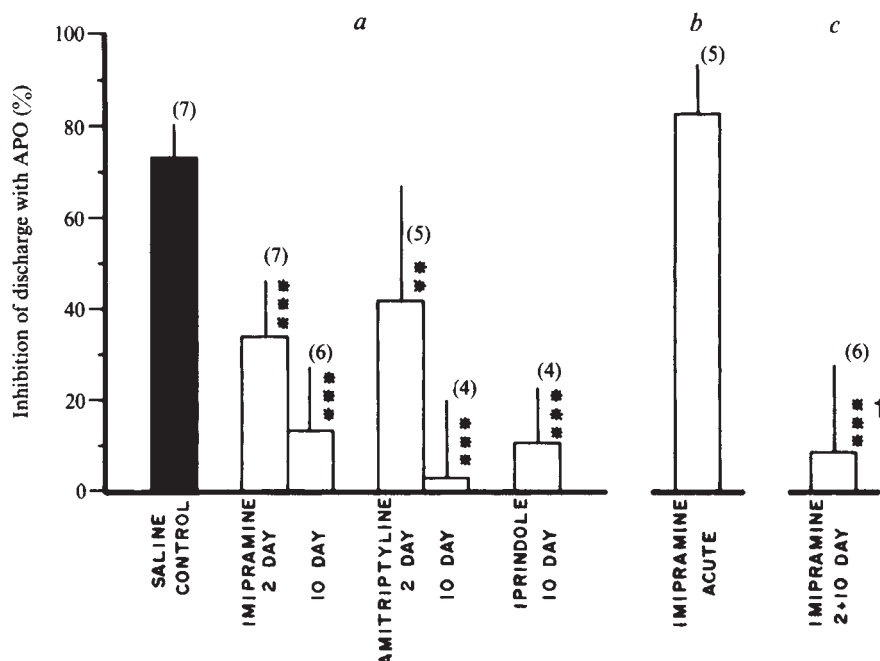


Fig. 1 The effects of various TCA treatments on the apomorphine-induced (APO; 0.004 mg per kg, i.v.) inhibition of the spontaneous activity of zona compacta DA neurones. See text for doses and details. *** $P < 0.001$, ** $P < 0.02$; two-tailed t -test, † $P < 0.05$ relative to 2-day imipramine group. Number tested in parentheses.

site was marked by passing a 40- μ A negative current for 10 min which results in the injection of the dye⁷. The animals were then perfused and the brain sliced and stained. All neurones reported in this study were histologically located in the zona compacta region of the substantia nigra.

Single DA neurones were located on the basis of the previously described electrophysiological criteria of Bunney and Aghajanian⁸. That is, DA cells within the SNC spontaneously discharged at rates of 1–9 Hz, had biphasic action potentials (+/–) with amplitudes of 0.4–1.5 mV and typically displayed a train of action potentials or ‘burst’ on discharge. All cells also met the pharmacological criteria for identifying DA neurones established by these investigators^{8,9}. Spontaneous activity was inhibited by the administration of a presynaptic dose of apomorphine and increased by the DA antagonist, haloperidol. To avoid contaminating any of our results, verification of the effects of haloperidol always took place immediately before terminating the recording session.

In the first experiment we investigated whether repeated injections of both typical (imipramine and amitriptyline) and atypical (iprindole) TCAs could alter DA autoreceptor sensitivity. Animals were placed into one of the following treatment groups: (1) 2 days of imipramine; (2) 10 days of imipramine; (3) 2 days of amitriptyline; (4) 10 days of amitriptyline; (5) 10 days of iprindole; and (6) 10 days of saline vehicle. All drugs were administered intraperitoneally in a dose of 10 mg per kg twice daily. Forty-eight hours after cessation of drug treatment the animals were recorded from as described above. Once a DA neurone was located its spontaneous activity was monitored for a minimum of 4 min to establish a reliable baseline rate. Apomorphine was then administered. Since a 0.004 mg per kg (i.v.) dose of apomorphine has been shown to inhibit DA neuronal activity by stimulating only DA autoreceptors located within the SNC¹⁰, we used the degree of inhibition obtained with this dose as our index of DA autoreceptor sensitivity. The present results indicate that repeated TCA injection induces a subsensitivity of presynaptic DA autoreceptors (Figs 1a, 3). All TCA treatments resulted in a significant attenuation of the ability of apomorphine to inhibit the spontaneous discharge of DA neurones. Ten days of TCA administration resulted in a greater degree of DA autoreceptor subsensitivity than 2 days of treatment. Although TCA treatment also produced a tendency toward an overall increase in baseline firing rates, relative to saline controls this difference was not statistically significant.

To determine the possible functional significance of TCA-induced DA autoreceptor subsensitivity we examined the responsiveness of subsensitive cells to tail pressure, a mildly activating, non-painful stimulus. We have previously shown that tail pressure induces DA-dependent behaviours and also alters the activity of midbrain DA neurones^{5,6}. All TCA treatments increased the ability of tail pressure to alter the spontaneous discharge of DA neurones (Fig. 2). This effect was significantly different from saline controls in both the 2- and 10-day imipramine groups as well as the 10-day amitriptyline group ($P < 0.05$ for all three). Interestingly, the 10-day amitriptyline group, which showed the greatest degree of DA autoreceptor subsensitivity, was the group most responsive to tail-pressure induced changes in discharge. The fact that some of the groups (that is, the 10-day iprindole) did not reach statistical significance is probably due to a combination of relatively small group size and a large degree of group variance.

To investigate the possibility that residual TCAs might simply be competing with apomorphine for autoreceptor binding and, in so doing, reduce its effectiveness, we examined the effects of acute administration of imipramine (0.4 mg per kg, i.v.) on the subsequent injection of apomorphine (0.004 mg per kg, i.v.). Acute imipramine had no effect on the ability of apomorphine to inhibit DA neuronal discharge (Fig. 1b). It also failed to alter the spontaneous activity or responsiveness to tail pressure of these neurones relative to controls (Fig. 2).

The augmentation of DA autoreceptor subsensitivity seen after 10 days of imipramine and amitriptyline treatment is reminiscent of the progressive behavioural sensitization that can occur following the administration of amphetamine and other stimulants^{11,12}. Although stimulant sensitization is usually induced by repeated drug treatment it is not dependent on such treatment and long-lasting effects have been observed following only a single amphetamine injection¹³. These findings raise the question of whether the progressive DA autoreceptor subsensitivity that occurs between 2 and 10 days of TCA treatment actually depends on administering these agents for 10 days.

To answer this question we ran an additional group of animals which received 2 days of imipramine (10 mg per kg, i.p. twice a day) followed by 10 drug-free days. Thus, this group was tested for subsensitivity at the same time as the group receiving 10 days of imipramine followed by 2 drug-free days. This treatment produced the same degree (actually 32% greater) of DA autoreceptor subsensitivity as the 10-day imipramine group

(Fig. 1c). Consistent with these data, 2 days of imipramine followed by 10 drug-free days (2+10 day group) resulted in significantly greater subsensitivity than an equal number of injections followed by only 2 drug-free days (2+2 day group). That is, the administration of the presynaptic dose of apomorphine inhibited the spontaneous activity of DA cells by only $9.2 \pm 19.0\%$ in the 2+10 day group but by $34.1 \pm 12.4\%$ in the 2+2 day group ($P < 0.05$, see Fig. 1). This experiment clearly demonstrates that the TCA-induced progression in DA autoreceptor subsensitivity is not dependent on repeated daily drug treatment. Moreover, these data provide an additional and definitive argument against explaining subsensitivity in terms of direct competition between TCAs and apomorphine. That is, maximal subsensitivity was observed at a time (10 days after cessation of injections) when little if any residual imipramine would be expected in the brain.

The present study provides the first electrophysiological demonstration of DA autoreceptor subsensitivity. Moreover, the fact that both classical and newer TCAs induce progressive subsensitivity within the same time frame as their antidepressant effects suggests that DA autoreceptor subsensitivity must be considered as a possible mechanism underlying the therapeutic effects of these agents. Finally, and perhaps most significantly, we have found that the progressive augmentation of DA autoreceptor subsensitivity induced by TCAs is not dependent on repeated daily drug administration. Thus, short-term drug treatment seems to be sufficient to trigger a subsensitivity which then undergoes a progressive enhancement as a function of time regardless of whether TCAs are again administered. This finding may be clinically relevant in several respects. If presynaptic DA autoreceptor subsensitivity is critical for the therapeutic efficacy of TCAs, then our data suggest that daily treatment with these agents may not be necessary for the amelioration of depression. The same data also provide a possible explanation of the delayed therapeutic onset of TCAs—it may reflect the time necessary for the development of a critical degree of receptor subsensitivity as well as the build-up of TCAs to steady-state therapeutic concentrations¹⁴. It would be interesting to know whether TCA-induced changes in noradrenaline^{15,16} and serotonin¹⁷ receptors are also independent of repeated drug treatment.

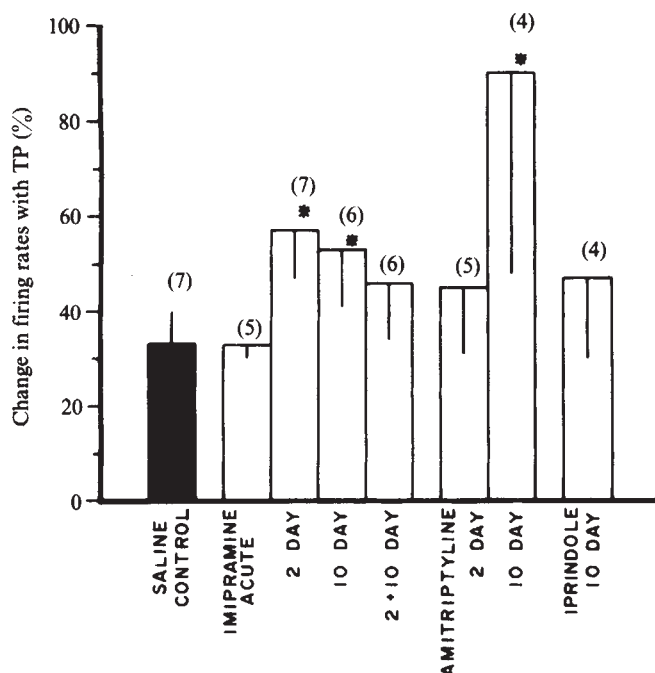


Fig. 2 TCA potentiation of tail-pressure induced (TP) changes in DA neuronal discharge. Data from same animals represented in Fig. 1. * $P < 0.05$; two-tailed t -test. Number tested in parentheses.

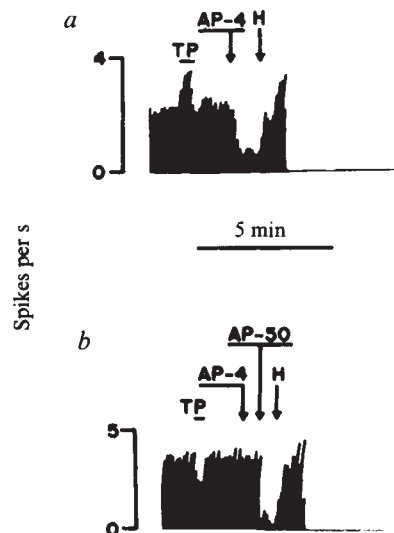


Fig. 3 Cumulative histograms of DA neurones located in the zona compacta demonstrating the effects of repeated TCA treatment on the ability of a presynaptic dose of apomorphine (APO-4; $4 \mu\text{g}$ per kg, i.v.) to inhibit the spontaneous firing of these neurones. *a*, Saline-treated control cell. A presynaptic dose of apomorphine produced an 81% inhibition of neuronal activity. Haloperidol (HAL; 0.2 mg per kg, i.v.) reversed this effect. *b*, Cell from an animal treated with imipramine for 10 days. A presynaptic dose of apomorphine had no effect on the spontaneous activity of this cell however, the subsequent administration of a postsynaptic dose of apomorphine (APO-50; $50 \mu\text{g}$ per kg, i.v.) was able to inhibit neuronal discharge by 86%. Haloperidol (0.2 mg per kg, i.v.) again reversed this effect. Horizontal bars represent the duration of the sensory stimulus, mild tail pressure (TP). Arrows indicate the i.v. administration of pharmacological agents.

The possible functional significance of subsensitive DA autoreceptors is reflected in our finding that animals showing such a subsensitivity also demonstrate an enhanced responsiveness to an activating environmental stimulus (tail pressure). Enhanced responsiveness to environmental stimuli is essentially the functional definition of successful TCA treatment.

Although TCA doses used in these experiments are about four times those used clinically this is not likely to explain our findings as preliminary data from our laboratory indicate similar effects with electroconvulsive shock, a non-pharmacological antidepressant treatment. This raises the question of whether our findings are specific to treatments having an antidepressant effect. In this regard, it should be noted that perturbation of DA autoreceptors does not invariably give rise to subsensitivity. Using a similar procedure Gallager *et al.*¹⁸ have shown that repeated treatment with haloperidol induces DA autoreceptor supersensitivity.

We can only speculate about the mechanisms underlying TCA-induced subsensitivity of DA autoreceptors. First, a blockade of dendritic DA uptake¹⁹ would result in overstimulation of DA autoreceptors which might then compensate by the induction of subsensitivity. Second, by analogy to the finding that repeated TCA administration produces a decrease in the number of cortical β -adrenergic receptors¹⁶, it is possible that a similar decrease in the number of DA autoreceptors underlies our effects. Finally, it is important to note that our data are not incompatible with the induction of noradrenergic subsensitivity^{15,16} which has been reported to follow repeated TCA treatments. Our noradrenaline-dopamine interaction hypothesis^{20,21} indicates that decreased noradrenaline activity actually results in an increase in DA functioning. For instance, interference with noradrenaline activity either by inhibiting synthesis, stimulating presynaptic α -receptors or postsynaptic

α -receptor blockade can reverse the behaviourally suppressive effects of postsynaptic DA receptor antagonists (ref. 20, and unpublished observations).

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Interferon treatment inhibits glycosylation of a viral protein

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Earlier, we reported¹ a 30–200-fold reduction in the yield of infectious vesicular stomatitis virus (VSV) released from L cells treated with 10–30 reference units ml⁻¹ of interferon (IFN); however, in these cultures virus particle production, as measured by VSV particle-associated viral RNA, virus nucleocapsid protein and viral transcriptase, was inhibited less than 10-fold. There was biochemical and morphological evidence of a significant reduction in glycoprotein (G) and membrane protein (M) of VSV particles released from IFN-treated cells^{2,3}. We compare here the effects of tunicamycin (TM) and IFN in L cells. Treatment with TM or IFN reduced the production of infectious VSV particles, decreased the amount of G and M proteins in VSV released from treated cells, and inhibited an early step in the formation of asparagine-linked oligosaccharide chains, the incorporation by membrane preparations from treated cells of *N*-acetylglucosamine into glycolipids with the properties of dolichol derivatives.

It is known that G protein of VSV contains two 'complex' type asparagine-linked oligosaccharide chains⁴. The role of polyisoprenoid glycolipid intermediates in the biosynthesis of *N*-glycosidically bound oligosaccharides is well established^{5–8}, and work by several groups^{9–11} has implicated dolichol-linked oligosaccharides in the glycosylation of the G protein of VSV. TM,

a glucosamine-containing antibiotic produced by *Streptomyces lysosuperficus*¹², inhibits the lipid intermediate pathway by blocking the transfer of *N*-acetylglucosamine-1-phosphate from UDP-*N*-acetylglucosamine to dolichyl monophosphate (Dol-P)^{13–19}. Treatment with TM inhibited the formation of glycosylated G protein in VSV-infected BHK cells^{20–22}.

The Indiana strain of VSV used in this work was plaque purified and passaged at low multiplicities. Virus titre was assayed as 50% tissue culture infectious doses (TCID₅₀) by observation of virus-induced cytopathic effect in Vero cells grown in microtitre plates, or by a plaque titration method as PFU ml⁻¹; the titre was 2 × 10⁹ TCID₅₀ ml⁻¹. The mouse L cell (from Dr D. Burke) was very sensitive to the virus growth inhibitory effect of mouse IFN. Mouse IFN was prepared and partially purified on an antibody affinity column²³. The specific activity of this preparation was greater than 4 × 10⁷ international mouse reference units (r.u.) per mg protein.

Table 1 Effect of IFN and TM on the production of VSV

VSV released from L cells treated with:	Virus titre (PFU ml ⁻¹)	Inhibition (log ₁₀)
Control for IFN (no treatment)	8.0 × 10 ⁷	—
IFN (30 r.u. ml ⁻¹)	7.5 × 10 ⁵	2.1
Control for TM (no treatment)	3.0 × 10 ⁷	—
TM (0.5 µg ml ⁻¹)	4.0 × 10 ⁶	0.90
TM (1.0 µg ml ⁻¹)	4.0 × 10 ⁵	1.90

L cells were grown in 150 cm² flask (2 × 10⁷ cells) and treated with 0–30 r.u. per ml of interferon for 14 h; the cell monolayers were washed three to four times to remove the residual interferon. All monolayers were then infected with VSV at a multiplicity of five plaque-forming units (PFU) per cells. TM (0.5 or 1 µg ml⁻¹) was added to cells after 1 h of VSV infection. All monolayers were further incubated for 20 h. The supernatant fluids were then collected and sedimented at 10,000g to remove cell debris. Virus titre in these supernatant samples were quantified by a plaque titration method.

There was a 200-fold decrease in infectious virus titre in VSV released from L cells treated with IFN (30 r.u. ml⁻¹). TM (0.5–1 µg ml⁻¹) inhibited the infectious virus production in the range of 10–80-fold (Table 1). Purified virus samples with the same amount of protein or radioactivity were applied to SDS-polyacrylamide gel electrophoresis (PAGE) slab gels²⁴. After electrophoresis, the gels were stained, dried and exposed to Kodak X-OMAT film. When the incorporation of radioactive precursor was quantified by fluorography²⁵, three proteins (G, N and M) were clearly visible in samples of virus not treated with TM; however, in VSV released from TM (0.5–1 µg ml⁻¹)-treated cells there was a dose-dependent decrease in G protein; also, a faster moving band appeared between G and N proteins. This band could not be labelled by glucosamine, suggesting that this was an unglycosylated G protein (Fig. 1, lane 3); the M protein was also inhibited in samples treated with TM. In VSV released from IFN-treated cells, there were also three proteins (G, N and M) clearly visible; however, the G and M proteins were reduced in amount compared to VSV released from cells not treated with IFN (Fig. 1, lane 7). When the G protein was labelled with glucosamine and an equal amount of protein loaded on gels, we observed that in VSV released from IFN-treated cells, there was a very significant inhibition of glucosamine incorporation. Electron microscopic studies revealed that VSV particles released from cells treated with either TM or IFN lacked glycoprotein spikes. These effects of TM on released VSV were very similar to those observed in VSV released from cells treated with IFN (30 r.u. ml⁻¹) and suggested that in VSV released from cells treated with IFN, there was a deficiency in G protein compared to VSV released from untreated cells.

Since lipid-linked oligosaccharides are involved in the glycosylation of VSV glycoprotein G^{9–11}, we carried out preliminary studies to see if IFN treatment affected the biosynthesis

of *N*-acetylglucosaminyl derivatives of dolichol. When membranes from IFN-treated cells were incubated with UDP-*N*-acetyl-[14 C]glucosamine, only 18% as much label was incorporated into glycolipid as compared to the preparations from untreated cells (Table 2). When the assays were carried out in the presence of 10 nmol of exogenous Dol-P, the labelling of glycolipid was stimulated in both preparations, but the rate of synthesis of glycolipid was still substantially higher in membranes from untreated cells. This result indicated that the reduction in glycolipid formation by IFN-treated cells was probably not due to a deficit in endogenous acceptor lipid. The direct addition of IFN to the incubation mixture had no effect on

the labelling of the glycolipid fraction. The observations that the labelling of *N*-acetylglucosaminyl lipid was stimulated by exogenous Dol-P, was inhibited by TM¹³⁻¹⁸ (data not shown), and that *N,N'*-diacetyl-[14 C]chitobiose was released by mild acid (0.01 M HCl, 100 °C, 15 min) treatment of the glycolipid fraction, strongly suggest that the glycolipids labelled in these experiments were *N*-acetylglucosaminyl derivatives of dolichol involved in lipid-mediated protein glycosylation. More detailed studies on the characterization of the glycolipids synthesized by this *in vitro* system are in progress.

Inhibition of the synthesis of *N*-acetylglucosaminyl lipids with properties of dolichol derivatives, that serve as precursors for the dolichol-bound oligosaccharide lipid intermediates in protein glycosylation, was lower by membrane preparations from IFN-treated cells as compared to membrane preparations from untreated cells and could also be partially responsible for results obtained with VSV released from L cells treated with IFN; such virions were selectively deficient in G and M protein¹⁻³. It might also explain results on Moloney murine leukaemia virus (MMLV) from IFN-treated TB cells, where virus with markedly decreased infectivity was produced²⁶. The MMLV virions were also deficient in the viral envelope glycoproteins gp 69/71, so that the decreased amount of glycoprotein may be directly related to a slower rate of synthesis of *N*-acetylglucosaminylpyrophosphoryldolichol (GlcNAc-P-P-Dol) in IFN-treated cells.

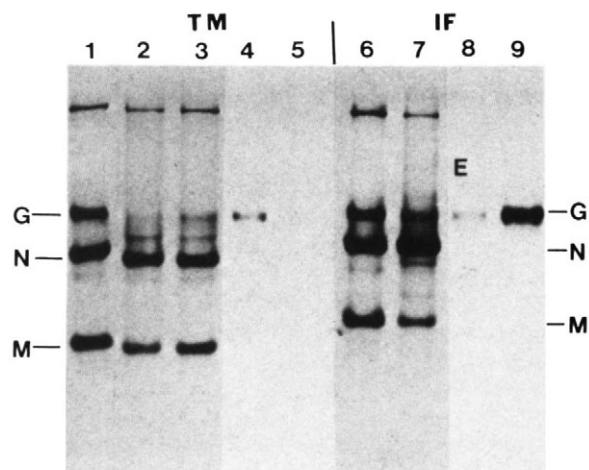


Fig. 1 L cells were treated with IFN or TM as described in Table 1. After virus adsorption and incubation for 2 h, the monolayers were washed with methionine-free medium and then 35 S-methionine (20 μ Ci ml⁻¹, specific activity, 800 Ci mmol⁻¹, Amersham) was added to monolayers in special minimal essential medium (MEM) devoid of cold methionine and supplemented with 2% dialysed fetal calf serum (FCS). After 6 h of incubation, 2 ml of regular MEM supplemented with 5% FCS was added to each culture and cells were incubated overnight. For labelling of glycoprotein (G), monolayers were infected with VSV for 2 h and the cultures were washed with glucose-free MEM. All cultures were refed with 15 ml of special medium (glucose-free MEM) containing 20 Ci ml⁻¹ of 3 H-glucosamine for 6 h and then 2 ml of regular MEM was added to each culture and cultures were incubated overnight. The supernatant fluids were collected and clarified at 10,000g for 15 min to remove cellular debris. The clarified supernatant fluids were centrifuged at 42,000g for 2 h. The pellet was resuspended in a small volume of TES (Tris-EDTA-sodium chloride) buffer (pH 7.2) and was further purified by banding on 20–50% (w/w) equilibrium sucrose gradients for 16 h at 72,000 g. Proteins were analysed by SDS-PAGE²⁴. Purified VSV proteins with equal amount of radioactivity were applied to the gels except those in which G protein was labelled with 3 H-glucosamine where equal amounts of protein were added. The gels were subjected to electrophoresis and then stained with Coomassie brilliant blue R. After destaining they were impregnated with 2, 5-diphenyloxazole (NEN), dried, and exposed to Kodak X-OMAT X-Ray film. Phosphorylase B (molecular weight 92,500), bovine serum albumin (molecular weight 69,000), ovalbumin (molecular weight 46,000), carbonic anhydrase (molecular weight 30,000), and cytochrome c (molecular weight 12,300) were used as reference protein molecular markers. TM lanes: (1), proteins labelled with 35 S-methionine of VSV from cells not treated with TM; (2), proteins of VSV from cells treated with TM (1 μ g ml⁻¹); (3), proteins of VSV from cells treated with TM (0.5 μ g ml⁻¹); (4), G protein of VSV labelled with 3 H-glucosamine and released from cells not treated with TM; (5), G protein of VSV released from cells treated with TM (1 μ g ml⁻¹). IFN lanes: (6), proteins labelled with 35 S-methionine of VSV from cells not treated with IFN; (7), proteins of VSV from cells treated with IFN (30 units ml⁻¹); (8), G protein of VSV labelled with 3 H-glucosamine and released from cells treated with IFN (30 r.u. ml⁻¹); (9), G protein of VSV released from cells not treated with IFN. Three VSV proteins, G, N and M, are visible on gels.

Table 2 Effect of IFN treatment on transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine into glycolipid

Treatment of cells	Addition to incubation mixture	<i>N</i> -Acetyl-[14 C]glucosamine incorporated into glycolipid fraction (c.p.m. per mg membrane protein)
None	None	6,180
None	Dol-P(10 nmol ⁻¹)	20,700
IFN(30 r.u. ml ⁻¹)	None	1,130
IFN (30 r.u. ml)	Dol-P(10 nmol)	2,680

L cells were grown in Petri plates to confluency (1×10^7 cells) and were treated with 30 r.u. per ml of interferon overnight. All cell monolayers were washed with saline three times, scraped, and centrifuged at 1,500 r.p.m. for 10 min. The cell pellets were suspended in 4 vol of ice-cold buffer consisting of 0.1 M Tris-HCl (pH 7.0), 0.25 M sucrose, 1 mM EDTA and homogenized. After homogenization, the suspensions were clarified by centrifugation at 8,500g for 5 min. The supernatant fluids were pelleted at 39,000g for 20 min and the pellets (crude particulate fraction) were resuspended in homogenization buffer. The protein content was measured by the Lowry procedure²⁸. Each reaction mixture contained crude particulate enzyme preparation; 50 mM Tris-HCl (pH 8.0); 0.125 M sucrose; 0.5 mM EDTA; 15 μ M UDP-*N*-acetyl-[14 C]glucosamine (620 c.p.m. pmol⁻¹); 10 mM MgCl₂ and 0.02% Triton X-100 in a total volume of 0.1 ml. Dolichyl monophosphate was dispersed by ultrasonication in 0.1% Triton X-100 and 10 nmol were added where indicated. Following an incubation at 37 °C for 10 min the amount of *N*-acetyl-[14 C]glucosamine incorporated into the glycolipid fraction was assayed as described elsewhere¹⁷. Lipid-phosphorous was determined by the Bartlett procedure²⁹. Dol-P, dolichyl monophosphate.

It will be interesting to determine whether the synthesis of GlcNAc-P-P-Dol is generally sensitive to IFN-treatment in a wide variety of animal cells. If glycosylation of asparagine residues in viral membrane glycoproteins is critical for the assembly of infectious particles of some viruses, inhibition of the enzymatic transfer of *N*-acetylglucosamine-1-phosphate from the nucleotide derivative to Dol-P would be an effective means of impeding viral production. The effects on glycosylation may also be pertinent to the varied activities of interferons on cell growth and on the immune system²⁷.

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Integration of Moloney leukaemia virus into the germ line of mice: correlation between site of integration and virus activation

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Endogenous avian^{1–3} and murine^{4–9} C-type viruses are genetic elements which are transmitted at several distinct chromosomal loci. Different patterns of virus activation have been observed in a variety of different mouse strains^{10–12}. However, because there are multiple copies of closely related endogenous viruses in every mouse strain, the genetic basis of the differential expression of these genes is poorly understood. A new substrain of mice, BALB/Mo, was derived previously¹³ carrying the exogenous Moloney leukaemia virus (M-MuLV) genome on chromosome 6 (ref. 14). Virus activation occurs in lymphatic tissues of all BALB/Mo mice soon after birth¹². We report here the derivation of three new substrains of mice each carrying a single M-MuLV genome on different chromosomal integration sites. Each locus was associated with a distinct phenotype of virus expression. Evidence is presented that apparently identical viral genomes show differences in spontaneous virus activation and that defective viral genomes can be carried in the germ line of mice.

Four to eight cell embryos were isolated from pregnant mice, treated with pronase to remove the zona pellucida and cultivated *in vitro* as described previously^{13,15}. For infection with M-MuLV the embryos were co-cultivated overnight with subconfluent CI 1A cells¹⁶. They developed into blastocysts and were subsequently transferred into the uterus of a pseudopregnant female. Of 28 mice derived in this way, 12 were viraemic when tested by radioimmunoassay for p30 at the age of 4 weeks. One viraemic male (no. 62) of ICR origin was selected. When mated with normal females, 20 of his 75 offspring were viraemic at 4 weeks of age. This paternal transmission of virus suggests that M-MuLV was integrated into cells of the germ line of male

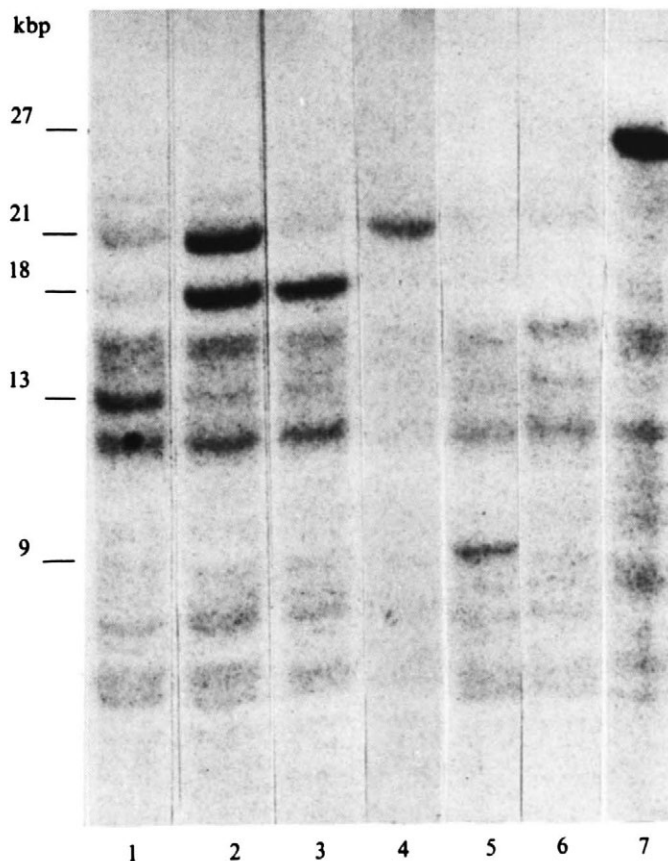


Fig. 1 Restriction enzyme analysis of mouse liver DNA. High molecular weight DNA from liver biopsies was digested with *EcoRI*, separated on agarose gels, transferred to nitrocellulose filters and hybridized to M-MuLV-specific cDNA as described¹⁹. *EcoRI*-digested λ DNA was used as a size marker. Lane 1, DNA from male 62; lanes 2–5, DNA from individual N-1 animals; lane 6, ICR control; lane 7, BALB/Mo DNA. The size and position of M-MuLV-specific fragments is indicated on the left. kbp, Kilobase pairs.

62. The fraction of viraemic offspring observed (26%) deviates from the mendelian expectations for transmission of a single dominant gene and suggests 'germ-line mosaicism'¹³. Biochemical evidence (Fig. 1) indicates that male 62 was not only mosaic in the germ line but also in somatic tissues (see below). In contrast, his viraemic sons transmitted viraemia to approximately 50% of their offspring. This mendelian transmission indicates that they were heterozygous for a single viraemia-inducing genetic locus and indicates that the exogenous M-MuLV had been converted to an endogenous virus in these mice.

The integration site of M-MuLV in the DNA of male 62 and his offspring was analysed with the restriction enzyme *EcoRI* using the technique of Southern¹⁷. This enzyme has no recognition site in the viral genome¹⁸. M-MuLV-specific sequences were detected above the background of endogenous murine viruses by using an M-MuLV-specific probe¹⁹. Figure 1, lane 7, shows a single M-MuLV-specific fragment with a molecular weight of 27 kilobase pairs in BALB/Mo liver DNA which was run as a control. This band represents the endogenous M-MuLV copy in BALB/Mo mice^{19,20}. Multiple bands with much lower relative intensities than the M-MuLV-specific bands are present in all mouse DNAs and are due to residual cross-homology of our specific probe with endogenous murine viruses. Three new M-MuLV-specific fragments not present in the uninfected control (lane 6) with molecular weights of 21, 18 and 9 kilobase pairs, respectively, were detected in the offspring of male 62 (lanes 2–5). The liver of male 62 (lane 1) carries all three

Table 1 Characterization of genetically transmitted M-MuLV sequences and phenotype of mice

Origin	No. of animals	M-MuLV in <i>Eco</i> RI fragment (size in kilobase pairs)	No. of M-MuLV copies per diploid genome equivalent	Expression of viraemia	Genetic locus
Male 62	1	21 18 13 9	2	+	—
N-1 of male 62	3	21	1-1.2	—	<i>Mov-2</i>
	5	18	0.9-1.2	+	<i>Mov-3</i>
	8	21	2-2.4	+	<i>Mov-2</i>
		18		+	<i>Mov-3</i>
	6	9	0.9-1.1	—	<i>Mov-4</i>
	8	—	0	—	—
BALB/Mo (heterozygous)	—	27	1	+	<i>Mov-1</i>

Backcross animals of the N-1 generation from male 62 were derived as described in the text. Viraemic and non-viraemic offspring were partially hepatectomized²¹ and the liver DNA was subjected to restriction enzyme analysis and to quantitative hybridization using specific M-MuLV cDNAs as hybridization probes^{19,22}. The size of M-MuLV-specific *Eco*RI fragments (see Fig. 1) and the number of M-MuLV-specific DNA copies per diploid mouse genome equivalent are given²². Animals were tested for viraemia at 3 weeks of age¹³. Designation of genetic loci is explained in the text. Data on BALB/Mo mice are taken from previous publications^{19,20}.

M-MuLV-specific bands found in his offspring and one additional band of 13 kilobase pairs. These band intensities are weaker than the respective bands in DNA of his offspring. Together with quantitative hybridization data (Table 1) detecting only two M-MuLV copies per genome equivalent, these results indicate that M-MuLV sequences were present in only part of the liver cells. Thus male 62 showed mosaicism in somatic tissues as well as in the germ line.

Restriction enzyme analysis of liver DNA revealed five genotypes among his offspring (Table 1). The 21- and 18-kilobase pairs M-MuLV-specific bands segregated independently and were found alone or in combination in individual offspring. This suggests that a single blastomere of male 62 was doubly infected with virus and gave rise to a clone of infected cells. Animals carrying the 9-kilobase pair band presumably originated from germ cells derived from a different infected blastomere. This interpretation is supported by the equal intensities of the 21- and 18-kilobase pair bands as opposed to the unequal intensities of the 13- and 9-kilobase pair bands, respectively, in the liver of male 62 (Fig. 1, lane 1).

Genetic segregation studies showed that the 21- and 18-kilobase pair fragments were transmitted at the expected mendelian ratio of approximately 50% to the N-2 generation (Table 2). Germ-line transmission was also found in animals segregating the 9-kilobase pair copy. Our results define three new genetic loci carrying M-MuLV-specific sequences, designated *Mov-2*, *Mov-3* and *Mov-4* corresponding to the M-MuLV-specific *Eco*RI fragments of 21-, 18- and 9-kilobase pairs length, respectively. The locus representing the M-MuLV gene in BALB/Mo mice has been designated previously as *Mov-1* (ref. 14).

The structures of the integrated M-MuLV genomes were analysed after digestion of cellular DNA with the restriction enzyme *Pvu*II (Fig. 2). Fragments *a*, *b*, *c*, *d* correspond to the 3' half, the *pol* region, the *gag* region and the *pol-gag* junction, respectively, of the M-MuLV genome²³. No difference was observed between the internal M-MuLV fragments generated from *Pvu*II cleavage of DNA from mice of genotype *Mov-1* (lane 2), *Mov-2* (lane 3) or *Mov-3* (lane 4). DNA from mice with genotype *Mov-4*, however, had lost the largest *Pvu*II fragment (lane 5), suggesting that the integrated M-MuLV in these mice contains a deletion in the 3' half of the viral genome. Further analysis with *Sac*I confirmed this conclusion (data not shown). Our results show that no major rearrangements in the M-MuLV genomes carried in the latter three substrains of mice have occurred and that partially deleted viral copies can be present in the germ line of mice.

Table 2 correlates the genotype of the mice with the status of viraemia. Three different phenotypes were observed: (1) early development of viraemia in animals carrying the *Mov-3* gene;

(2) no viraemia at 3 weeks of age but occasional activation of M-MuLV later in life in animals carrying the *Mov-2* gene; (3) no occurrence of viraemia in animals carrying the *Mov-4* gene. Viraemia in *Mov-3* animals occurred at age 3 weeks or younger and was followed by rapid development of fatal leukaemia at 2 to 4 months of age. The genetic transmission of the *Mov-3* gene correlates well with the occurrence of viraemia and supports the conclusion that the presence of this gene induces early virus expression. *Mov-2* animals, however, occasionally activate virus later in life. Table 2 shows that 14 out of 150 N-2 animals which were derived from *Mov-2* males bred with normal females developed viraemia at 2 to 4 months of age. As only 50% of the offspring are expected to carry the *Mov-2* gene, the incidence of virus activation is approximately 20%. When viraemic N-2 males were bred again with normal females, a similar incidence of viraemia in the N-3 generation was observed. In contrast to *Mov-2* animals, none of 60 offspring of *Mov-4* animals became viraemic.

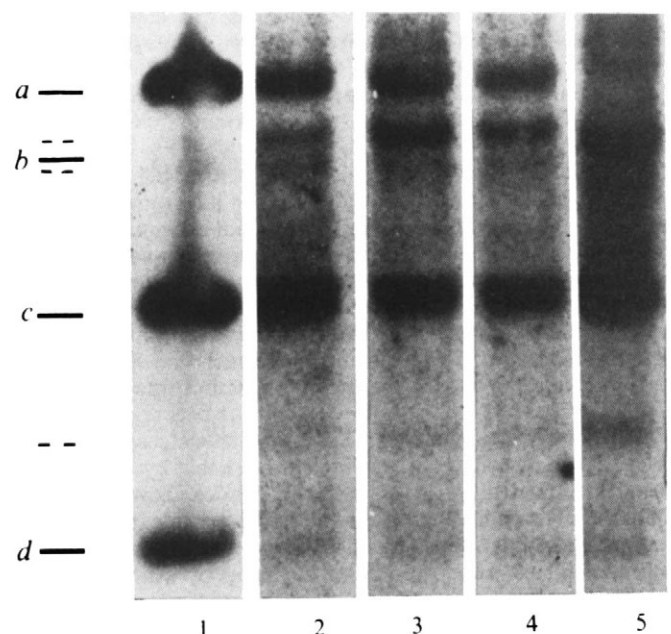


Fig. 2 Digestion of liver DNA by *Pvu*II. DNA was analysed as described in Fig. 1. Lane 1 shows a *Hind*III-*Pvu*II double digest of M-MuLV cloned in pBR 322 (provided by S. Goff). Lane 2, BALB/Mo DNA (*Mov-1*); lane 3, *Mov-2* DNA; lane 4, *Mov-3* DNA; lane 5, *Mov-4* DNA. *a-d* Mark the positions of the M-MuLV fragments after *Pvu*II digestion as published²³.

Table 2 Genetic transmission of M-MuLV sequences and incidence of viraemia in second backcross generation

M-MuLV-specific <i>Eco</i> RI fragment in father	Locus	M-MuLV-specific sequences in DNA of N-2 mice/ total tested	Viraemic N-2 animals/ total tested	Percentage
18 Kbp	Mov-3	14/28	125/255*	49
21 Kbp	Mov-2	6/14	14/147†	9.5
9 Kbp	Mov-4	1/5	0/60	0

N-1 males of genotype Mov-2, Mov-3 and Mov-4 were identified after partial hepatectomy as described in Table 1. They were bred with normal females and some of the offspring were again partially hepatectomized and the presence of M-MuLV-specific sequences in the liver DNA determined by quantitative hybridization²². All animals were repeatedly tested for viraemia using the RIA for p30 (ref. 13).

* Animals segregating the *Mov-3* locus were viraemic at the youngest age tested (3 weeks).

† Animals segregating the *Mov-2* locus were non-viraemic at 3 weeks of age; some mice developed viraemia at later ages (between 2 and 4 months of age).

Having become viraemic, Mov-2 animals develop leukaemia quickly. The disease is indistinguishable by histological and molecular parameters from the leukaemia occurring in Mov-3 animals. The host range of the activated virus was determined. The virus grew equally well on NIH 3T3 and BALB 3T3 cells but did not replicate in CCl 64 cells. This phenotype bred true when cloned on either of the permissive cell lines. Thus the virus was NB tropic like the original virus used for infection of the embryos.

The sequence homology of Mov-2 and Mov-3 virus was analysed by molecular hybridization. RNA was isolated from NIH 3T3 cells which were infected with Mov-2 virus and from spleens of viraemic Mov-2 and Mov-3 animals. Each RNA preparation annealed to the same extent (95%) to M-MuLV cDNA at low C_{t} values. In contrast, RNA of spleens from non-viraemic Mov-2 animals annealed to the same extent as RNA from control animals (15% annealing at a C_{t} value of 10,000). Furthermore, when DNA from NIH 3T3 cells infected with virus activated from Mov-2 animals was digested with *Pvu*II, no differences in the cleavage pattern of integrated viral sequences were observed in comparison to Cl 1A virus, BALB/Mo virus or Mov-3 virus (data not shown). This suggests that Mov-2 animals activate NB tropic Moloney virus integrated into their germ line.

We chose Moloney leukaemia virus as a model system to establish the experimental conditions for integrating genetic material into the germ line of mice. The animal exposed to virus was a mosaic carrying viral genomes in part of his somatic and germ cells and genetically defined mouse strains were only obtained after segregating the genes in the next generation. As preimplantation embryos are not permissive for expression of viral functions¹⁵, our experimental approach can be used to introduce other than viral genetic information into the germ line.

The M-MuLV genome carried at the *Mov-1*, *Mov-2* and *Mov-3* locus, respectively, was indistinguishable by all criteria used. Virus activation in the respective mice, however, was different. Thus, cellular sequences flanking an integrated viral genome may influence its expression by a *cis*-acting mechanism, as has been suggested^{24,25}. The mechanisms by which animals have acquired endogenous viruses and the evolutionary forces that influenced virus expression are not known. Our results indicate that the endogenous viruses of mice may have entered the germ line by infecting early embryos and that the different phenotypes of virus expression observed in inbred mouse strains may depend on the chromosomal integration site of the virus. Our model system should facilitate investigations to understand the regulation of C-type virus expression during cellular differentiation and its possible role in evolution.

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T-DNA of a crown gall teratoma is covalently joined to host plant DNA

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Agrobacterium tumefaciens strains containing tumour-inducing (Ti) plasmids^{1–3} incite cancerous growths called crown galls when inoculated into wounded dicotyledonous plants. Tumour tissue can be cultured axenically *in vitro*, and exhibits a transformed phenotype in the absence of the inciting bacterium. Transformed cells grow autonomously, are auxin and cytokinin autotrophic *in vitro*⁴ and synthesize opines^{5–8}, novel amino acid derivatives dictated by Ti plasmid genetic information^{9–11}. A small segment of the Ti plasmid, termed T-DNA is maintained in axenic tumour cells^{12–19}. Mitochondrial and chloroplast DNA from a crown gall teratoma are free from T-DNA, whereas nuclear DNA contains T-DNA in amounts similar to that in total tumour cell DNA^{20,21}. T-DNA appears to be attached to what is presumably plant DNA in the crown gall tumour cell: Southern blot analysis²² of tumour DNA digested with restriction endonucleases reveals T-DNA fragments that are not fully homologous to Ti plasmid DNA^{14,17,21}. We report here the isolation by molecular cloning of a 'border fragment' T-DNA and flanking plant DNA from the crown gall teratoma BT37 and show that T-DNA is covalently joined to a repeated DNA element of the tobacco nuclear genome.

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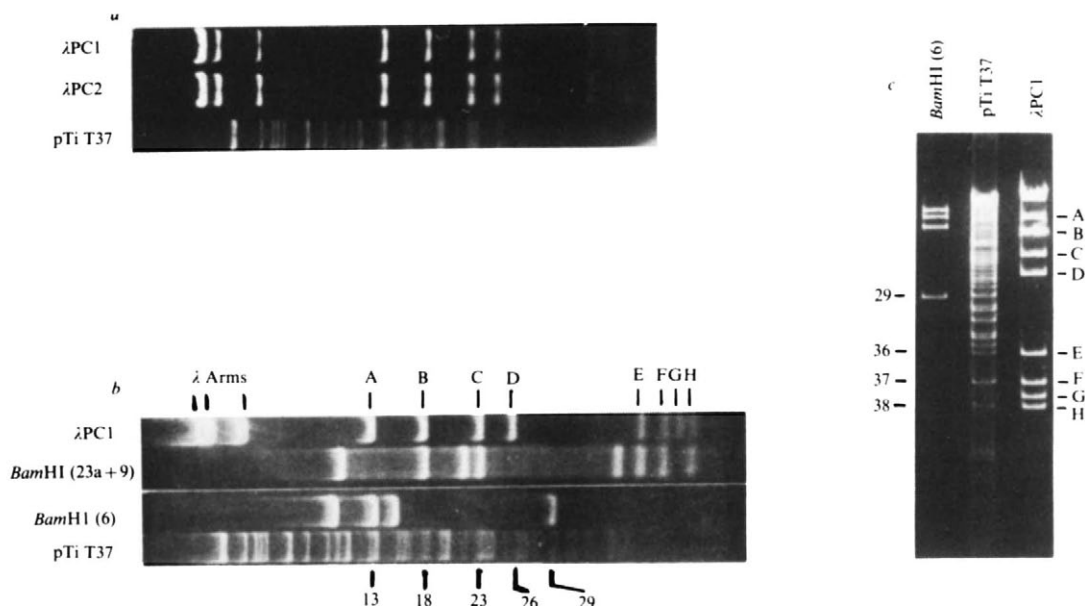


Fig. 1 *EcoRI* digests of DNA from λ PC1 and λ PC2, pTi T37 and cloned fragments of pTi T37. Charon 4A bacteriophage³⁰ DNA was digested with *EcoRI* and the vector arms separated from internal fragments by sedimentation through 5–20% NaCl linear gradients. Partial and complete *EcoRI* digests of BT37 tumour DNA, isolated as described in ref. 12, were fractionated in the same manner (F. Blattner, personal communication). Fragments 10–20 kilobases in size (correct for Charon 4A cloning inserts) were pooled. Phage arms (20 μ g) and target fragments (7 μ g) were incubated with T4 DNA ligase for 16 h at 4 °C (50 μ l reaction volume). Packaging was performed by published procedures^{23,24}. Phages were plated on 9.2 cm² Petri dishes at 20,000 plaques per plate. Lifts were prepared²⁵ and hybridized with pTi T37 DNA labelled *in vitro* by nick translation²⁶, in order to detect plaques containing T-DNA inserts. Two positive plaques, λ PC1 and λ PC2, were detected and purified. *a*, *EcoRI* digests of λ PC1 and λ PC2 DNA (isolated from CsCl banded phage particles by phenol deproteinization and dialysis) and of pTi T37 DNA were fractionated by electrophoresis in a horizontal slab gel of 0.7% agarose as described previously³¹. The three largest molecular weight fragments in the phage DNA digests are Charon 4A arms. *b*, *EcoRI* digests of λ PC1 DNA, pTi T37 DNA and two recombinant plasmids containing the left end of T-DNA from pTi T37 (see text and Fig. 2) were fractionated as described above in 1% agarose. *c*, *EcoRI* digests of λ PC1 DNA, pTi T37 DNA and (as molecular weight marker) one recombinant plasmid from the left end of T-DNA were fractionated by vertical 1.5% agarose slab gel electrophoresis in TB buffer (10.8 g Tris base, 0.93 g disodium EDTA and 5.5 g boric acid per litre). Fragments of pTi T37 were numbered starting from fragment 29, which is also indicated in *b*. Fragment G is not an internal pTi T37 fragment.

For molecular cloning of T-DNA fragments from BT37 tobacco teratoma DNA, we used the strategy of F. Blattner *et al.*²³: shotgun cloning of a mixture of *EcoRI* partial and complete digest fragments of total tumour DNA into Charon 4A bacteriophage. Ligated DNA (see legend to Fig. 1 for details) was packaged *in vitro* into phage coats^{23,24} and approximately 10⁶ plaque-forming units were plated and screened by hybridization of plaque 'lifts'²⁵ with Ti plasmid DNA, labelled *in vitro* by nick translation²⁶ with [α -³²P]deoxynucleotide triphosphates. Two positive plaques were picked and purified. The *EcoRI* cleavage patterns of the resulting two recombinant phage DNAs are presented in Fig. 1a. λ PC2 possesses six of the eight *EcoRI* insert fragments contained in λ PC1; the latter are designated A–H in decreasing order of molecular weight (Fig. 1b). To determine how many of the insert fragments were homologous to Ti plasmid DNA, a Southern transfer prepared from them was hybridized with ³²P-labelled²⁶ pTi T37 plasmid DNA. Autoradiography revealed that all insert fragments except the G fragment of λ PC1 hybridized strongly (data not shown). Further studies used only λ PC1 to determine whether any of its insert fragments were border fragments containing both Ti plasmid and plant DNA sequences.

A map of the part of pTi T37 detected in plant tumour line BT37^{16,19–21} is presented in Fig. 2. Part of *BstI* fragment 6 as well as the whole of 23A, 9 and 14A are detectable in the tumour DNA by Southern blot analysis²¹. Eight intact *EcoRI* fragments of the Ti plasmid are internal to this region and are also detectable by Southern blot analysis of BT37 tumour DNA^{19,21}. To compare the insert fragments of λ PC1 with the *EcoRI* fragments that map in T-DNA, we compared the single and multiple cleavage products of Ti plasmid fragments with

those of λ PC1. Because of the complexity of total Ti plasmid DNA, we used two clones of Ti plasmid DNA in the vector pBR322. One contained *BstI* fragment 6, and the other contained a partial digest product, *BstI* fragments 9 plus 23A plus the unnumbered small fragment between them. Figure 3 shows that *EcoRI* fragment 13 (cut from the appropriate *BstI* clone) co-migrates with A, 18 with B, and 23 with C. Also, the two subfragments of D generated by *BstI/EcoRI* double digestion of λ PC1 correspond to *BstI/EcoRI* double digest fragments of the appropriate *BamHI* clones (data not shown). Confirmation of D as *EcoRI* fragment 26 was achieved by recloning D into pBR325 (ref. 27), labelling this recombinant plasmid and hybridizing it to a Southern blot of pTi T37 *EcoRI* fragments as well as λ PC1 *EcoRI* fragments. The probe hybridized with D in the phage DNA digest as expected and with fragment 26, of identical electrophoretic mobility, in the Ti plasmid digest (data not shown).

The low molecular weight inserts E, F and H of λ PC1 co-migrate with subfragments of the (23A+9) *BstI* clone (Fig. 1b) and in the Ti plasmid *EcoRI* digest, fragment 36 co-migrates with E, 37 with F and 38 with H (Fig. 1c). Clearly fragment G is not an intact *EcoRI* fragment of the Ti plasmid: no *EcoRI* fragment of pTi T37 co-migrates with it (Fig. 1c).

Fragment G could be either a plant DNA fragment included in the original phage clone by coincidence, or a border fragment abutting A on the left or (less plausibly) F on the right. For DNA hybridization analysis, G was recloned into the *EcoRI* site of pBR325 (ref. 27), and an *EcoRI* digest of this clone, containing vector and G fragments, was used to prepare Southern blots. When duplicate tracks were hybridized with labelled pTi T37 DNA (Fig. 3a), fragment G hybridized weakly. G thus

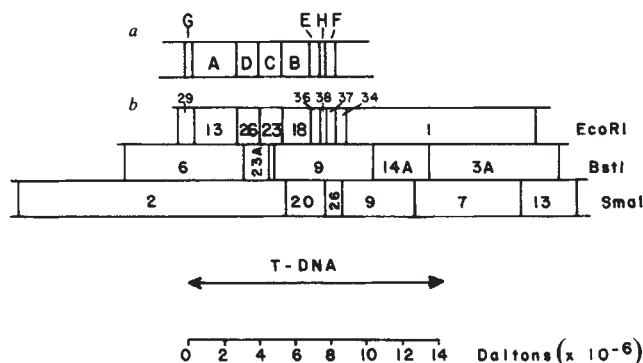


Fig. 2 Map of the T-DNA portion of pTi T37 and of the inserts of λ PC1. For the *Sma*I fragment map and evidence see ref. 16. The *Bst*I fragments were mapped hybridizing isolated *Sma*I and *Hpa*I fragments to Southern blots²² of *Bst*I digest fragments of pTi T37 as described previously³². *Bam*HI (a *Bst*I isoschizomer) fragments of partially digested pTi T37 were cloned into the *Bam*HI site of pBR322, and clones containing the following inserts were picked from a bank of 200 clones by colony hybridization³³: fragment 6, fragments 9 and 23A and the small unnumbered fragment between them, fragment 14A and fragment 3A. Digestion of these recombinant plasmids with *Sma*I, *Hpa*I, *Bst*EII and combinations of these enzymes produced the fragments predicted by the map presented here and the published *Hpa*I and *Bst*EII maps¹⁶. Digestion of these recombinant plasmids with *Eco*RI alone and in combination with the other enzymes mentioned above allowed us to determine the *Eco*RI fragment map of pTi T37 shown above. The numbers assigned to the small fragments (34–37) were determined by the method described in Fig. 1c legend. *a*, The fragment map of the inserts in λ PC1; *b*, the fragment map of pTi T37. Evidence for the arrangement of these fragments is based on the identification of their counterparts in the Ti plasmid digest as described in the text. Assignment of G to the left edge of the phage insert is based on its hybridization to Ti plasmid fragments mapping in this region (see text).

appeared to contain a short piece of Ti plasmid DNA. To determine whether G derived from the left edge of T-DNA, we hybridized Southern blots of Ti plasmid fragments generated by four different restriction enzymes (Fig. 3b). The G probe hybridized to *Bst*I fragment 6, *Eco*RI fragment 29, *Sma*I fragment 2 and *Hpa*I fragment 5 (see map in ref. 16), evidence that G is indeed from the left edge of T-DNA. *Eco*RI fragment 29

was recloned from *Bst*I fragment 6, and this recombinant plasmid also hybridized with G weakly (Figure 3a). We conclude that fragment G has weak homology with the leftmost edge of the T-DNA portion of the Ti plasmid. Much of G is not Ti plasmid DNA, for its hybridization with the Ti plasmid fragments is poor compared with homologous reactions run simultaneously.

Next we tested fragment G for homology with tobacco DNA. Normal tobacco leaf DNA and BT37 tumour DNA were cleaved with *Eco*RI or *Bst*I and the fragments immobilized by Southern blotting²². The cloned G fragment in the vector pBR325 was labelled by nick translation and used as probe. Figure 4 shows that this fragment hybridizes to a series of bands in the tobacco DNA digests. Two other fragments of λ PC1 (A and D) cloned in the same vector and used as probes in parallel hybridization studies with tobacco DNA did not hybridize to such bands (data not shown). We conclude that fragment G is homologous to a family of repeated DNA sequences in the tobacco genome. The prominent pattern of *Eco*RI fragments of tobacco DNA to which G hybridizes suggests that this family is a 'clustered repeat'^{28,29}. The smaller and simpler *Bst*I cleavage products to which G hybridizes suggest that there are *Bst*I sites internal to the repeat element. The role of such repeated DNA sequences in the eukaryotic genome is unknown. We note no significant difference in the intensity or pattern of bands in the tumour DNA digest compared with normal tobacco leaf DNA digest (Fig. 4 and other data not shown). Thus, the organization and copy number of the repeated DNA family to which G hybridizes is not altered grossly during tumorigenesis. Tumour DNA must contain one altered member of this family—the one into which T-DNA is inserted; however this member is not noticeable in Fig. 4, presumably because of the abundance of unaltered family members.

We conclude that the T-DNA found in BT37, a tobacco crown gall teratoma, is covalently joined at its left edge to plant DNA. Apart from this, the T-DNA fragments that we have isolated from this tumour line appear to be colinear with the corresponding region of the Ti plasmid: no noticeable alterations have occurred in this region of T-DNA whilst in the plant cell. Consistent with the location of T-DNA in the nuclear rather than chloroplast or mitochondrial DNA fraction²¹, its left border fragment hybridizes to repeated DNA characteristic of eukaryotic genomic DNA. It is not clear from this study whether T-DNA is an integral part of a tobacco chromosome in the transformed plant cell; it could constitute an extrachromosomal autonomously replicating DNA element. There is, however,

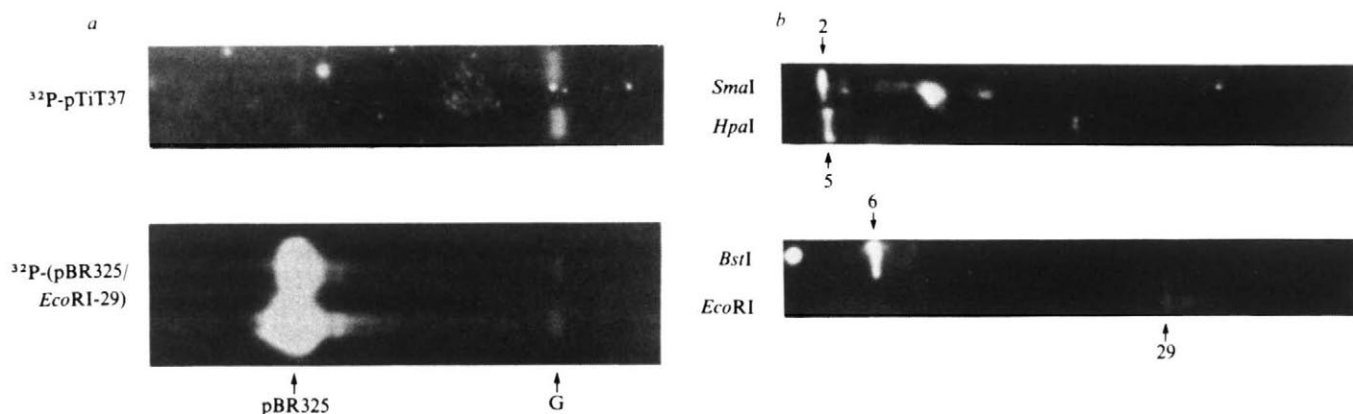


Fig. 3 Evidence for homology between fragment G and pTi T37. *a*, Southern blots containing fragment G and pBR325 (cut from the recombinant plasmid pBR325/*Eco*RI-G) were prepared in duplicate with the immobilized fragments at two loading levels (0.5 μ g for the upper track and 1.0 μ g for the lower track in each experiment). Blots were hybridized by the procedure of Thomashow *et al.*¹⁴ with either 1 μ g of pTi T37 DNA (2.6×10^7 c.p.m. μ g⁻¹) or 0.5 μ g of recombinant plasmid pBR325/*Eco*RI-29 DNA (1.66×10^8 c.p.m. μ g⁻¹) in 5 ml of reaction medium¹⁴. Probes were labelled by nick translation²⁶ with ³²P-labelled deoxynucleotide triphosphates. *b*, Southern blots²² of pTi T37 digests produced by four restriction endonucleases (*Sma*I, *Hpa*I, *Bst*I and *Eco*RI) were hybridized¹⁴ with 0.5 μ g of recombinant plasmid pBR325/*Eco*RI-G (1.26×10^8 c.p.m. μ g⁻¹). The numbers of the digest fragments to which the probe hybridized are indicated.

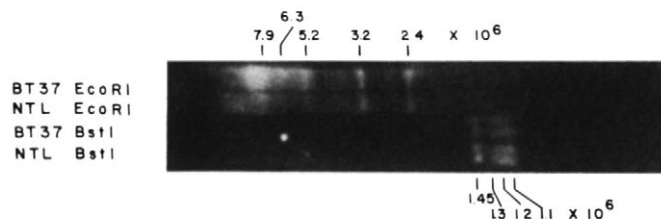


Fig. 4 Evidence for homology between fragment G and tobacco DNA. *Bst*I digests of 2.8 μ g of normal tobacco leaf DNA isolated as described elsewhere³⁴ and of BT37 tumour DNA, in addition to *Eco*RI digests of 5.0 μ g of the same two DNAs, were fractionated in horizontal slab gels of 0.7% agarose and blotted by Southern's procedure²². The blots were hybridized¹⁴ with 1.5 μ g of recombinant plasmid pBR325/*Eco*RI-G (5.4×10^7 c.p.m. μ g⁻¹). The autoradiogram shown was exposed for 48 h (with two Dupont Cronex intensification screens) at -80°C . Molecular weights of the bands in the autoradiogram were estimated from a calibration curve for the original gel based on digest fragments of known molecular weight. NTL, Normal tobacco leaf DNA, BT37, tobacco BT37 tumour DNA.

little precedent for the existence of such an element. Regardless of the physical situation of T-DNA our data make it clear that this foreign prokaryotic DNA element has become joined to eukaryotic DNA. The mechanism and specificity of such novel joining of phylogenetically alien DNAs may be revealed by analysis of DNA sequence at the border regions.

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Note added in proof: Similar results have been obtained for two T-DNA border fragments from an octopine-synthesizing tobacco crown gall tumour³⁵.

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Behaviour of octopus rhodopsin and its photoproducts at very low temperatures

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Many aspects of light energy transduction in photoreceptors are under study^{1,2}. One important problem concerns the light-initiated primary event in rhodopsin and the identity of the primary photoproduct. It is usually assumed that the red-absorbing pigment bathorhodopsin³ is the first photoproduct. In cattle rhodopsin, it is produced within 6 ps of excitation⁴ and is stable at 77 K. However, Yoshizawa *et al.*⁵⁻⁷ have found a blue-absorbing pigment, hypsorhodopsin, in photosteady-state mixtures formed with orange light at liquid helium temperatures (~ 5 K). On warming the hypsorhodopsin in the dark to liquid nitrogen temperatures (77 K), it converts to bathorhodopsin. These two results suggested that hypsorhodopsin might be a precursor of bathorhodopsin, but that presents difficulties. For example, hypso-type intermediates have not been seen for all visual pigments⁶. Moreover, when cattle rhodopsin was irradiated with blue light at liquid helium temperatures, it was converted to bathorhodopsin without forming detectable hypsorhodopsin^{6,7}. Finally, rapid kinetic experiments to determine the time course of formation of bathorhodopsin and hypsorhodopsin have given ambiguous results^{4,8-10}; this may be due to the use of rhodopsins from different species. Because squid rhodopsin seemed to have more hypsorhodopsin in photosteady states than cattle rhodopsin⁷, we have studied the photo products of another cephalopod rhodopsin at carefully controlled low temperatures (± 0.2 K). We report here that octopus rhodopsin has a hypso intermediate that is easily formed on irradiation but that the batho-product appears before significant amounts of the hypso-product accumulates. Moreover, the thermal conversion of hypsorhodopsin to bathorhodopsin must be fitted with two rate constants, and the activation enthalpy of the faster process is almost zero, that is, it is temperature independent over the range studied.

Octopus (*Mizudako*, *Paroctopus defleini*) microvillar membranes were prepared as previously described¹¹. Rhodopsin was extracted from the membranes with a 2% digitonin solution, glycerol was added to the preparation to give a final concentration of 75%, and the samples were stored at -70°C . The pH of the solution was adjusted to 10.5 with sodium carbonate buffer just before the start of an experiment, so that at room temperature any bleached rhodopsin would go to alkaline metarhodopsin ($\lambda_{\text{max}} = 376$ nm)¹².

On cooling to 10 K, the λ_{max} of octopus rhodopsin (476 nm at 10°C) shifted to 486 nm (Fig. 1, curve 1). When the octopus rhodopsin was very briefly irradiated with 480 nm light at 10 K, the spectral change indicated the formation of a bathochromic product (octopus bathorhodopsin) (Fig. 1, curve 2). On further irradiation, the concentration of the bathochromically absorbing product increases (curve 3) and the appearance of a hypsochromically shifted photoproduct (octopus hypsorhodopsin) can also be seen (Fig. 1, curve 4). With further irradiation using 530 nm light, a photosteady state is formed which contains large amounts of hypsorhodopsin (56%), as well as rhodopsin (<4%) and isorhodopsin (>40%), with almost no bathorhodopsin. A detailed analysis of the spectra shows that hypsorhodopsin and bathorhodopsin appear concomitantly. Figure 1 strongly suggests that on irradiation of rhodopsin at

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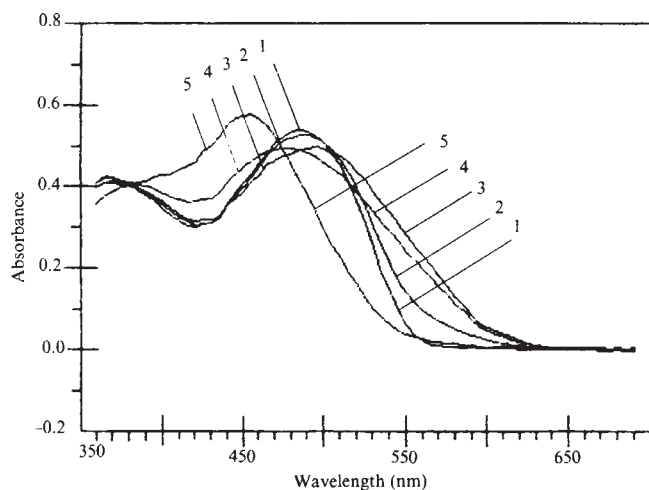


Fig. 1 Spectral changes observed in the conversion of rhodopsin to bathorhodopsin, hypsorhodopsin and isorhodopsin at 10 K. Curve 1, octopus rhodopsin in digitonin (pH 10.5) 10 K; curves 2-4, products of irradiation at 480 nm for successive periods of 8, 64 and 2,048 s; curve 5, photosteady state obtained by irradiation at 530 nm for 10 min. Curve 5 represents about 56% hypsorhodopsin, >40% isorhodopsin and <4% rhodopsin. This composition was determined as in ref. 7. All spectra were recorded with a Cary 14 using a Janis 10DT super Varitemp Optical Dewar.

10 K, the stable, blue-absorbing ground state species, hypsorhodopsin, is apparently not the precursor of the bathorhodopsin formed at this temperature.

On slowly warming up from 45 K, the spectrum of the photosteady-state mixture formed by irradiating octopus rhodopsin at 10 K shifted to longer wavelengths with sharp isosbestic points at 479 nm and 374 nm, as shown in Fig. 2. In the insert, the change in absorbance at 540 nm, indicating the formation of bathorhodopsin, is shown as a function of temperature. Judging from the similarity of their absorption spectra, the batho-product obtained by warming is identical to bathorhodopsin produced by irradiation of octopus rhodopsin at 80 K (B. Mao, M.T. and T.G.E., unpublished observations). Thus, the data of the insert show that hypsorhodopsin is converted to bathorhodopsin above 45 K. This transition temperature is much higher than that observed for the bovine⁶ and

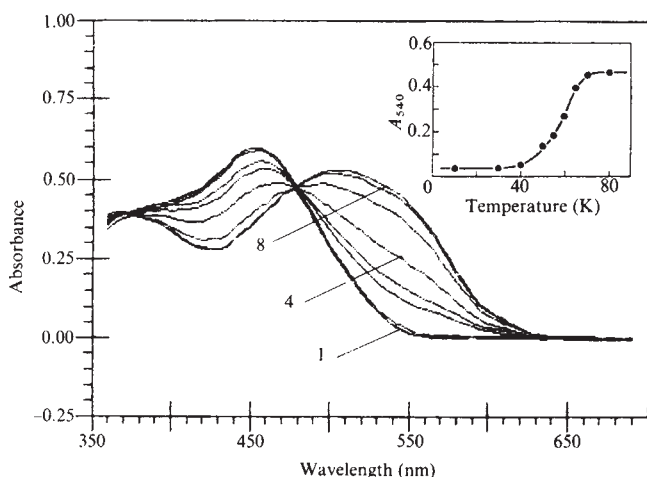


Fig. 2 The transformation of hypsorhodopsin to bathorhodopsin on warming above 10 K in the dark. The photosteady state (curve 1) was produced by irradiating rhodopsin at 10 K with 530 nm light for 5 min. The sample was then slowly warmed and spectra recorded every few minutes. The temperatures of the sample when the spectrum was measured are: (1) 10 K, (2) 40 K, (3) 50 K, (4) 55 K, (5) 60 K, (6) 65 K, (7) 70 K, (8) 80 K. Inset: absorbance at 540 nm (indicating the formation of bathorhodopsin) as a function of temperature.

squid⁷ or frog¹³ hypsorhodopsin → bathorhodopsin transition. There is no evidence of any stable intermediates between hypsorhodopsin and bathorhodopsin.

We attempted to measure the enthalpy of activation in the thermal conversion of hypsorhodopsin to bathorhodopsin. At a given temperature, rhodopsin was irradiated with continuous yellow light ($\lambda > 490$ nm) for 3 min to reach the photosteady state. Immediately after the light exposure, the decay of hypsorhodopsin was monitored by the absorbance at 440 nm and the formation of bathorhodopsin by the absorbance at 550 nm. For temperatures between 53 K and 73 K, we plotted $\ln(A_t - A_\infty)$ as a function of time, where A_t and A_∞ are the absorbance at t min and infinity, respectively. The rates of both hypsorhodopsin and bathorhodopsin are non-exponential; both curves can be fitted fairly well by the sum of two exponentials. The temperature dependence of the two rates is plotted in an Arrhenius manner ($\ln K$ against $1/T$) in Fig. 3. The temperature dependence of the decay of hypsorhodopsin gives $\Delta H = 0.87 \pm 0.29$ kcal mol⁻¹ and $\Delta H = 0.09 \pm 0.28$ kcal mol⁻¹. A

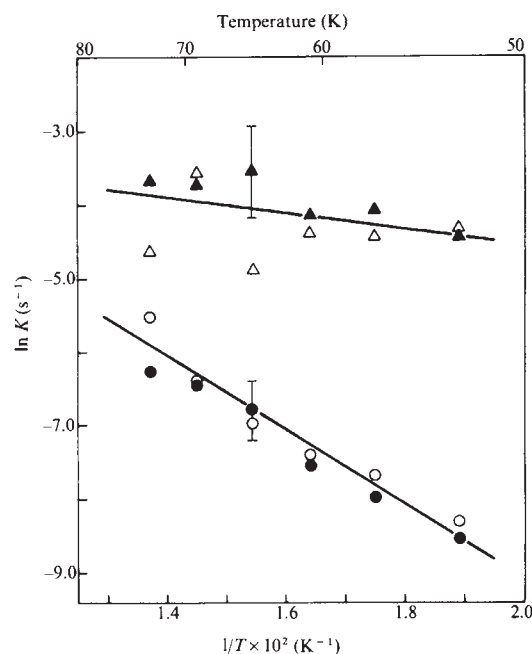


Fig. 3 Arrhenius plot of kinetic data for the decay of hypsorhodopsin (decrease in absorbance at 440 nm) and formation of bathorhodopsin (increase in absorbance at 550 nm). Δ , Faster phase; $\circ$, slower phase in the decay of hypsorhodopsin. $\blacktriangle$, Faster phase; $\bullet$, slower phase in the formation of bathorhodopsin.

similar pair of values are found for the formation of bathorhodopsin. The non-exponential character of the hypsorhodopsin decay processes implies that hypsorhodopsin is not a single homogeneous species. We are now studying the thermal decay process in more detail, in particular the nature of the component which seems to be almost temperature independent.

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Contrast perception above threshold is only minimally impaired in human amblyopia

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Amblyopia is a condition occurring at birth, or in early childhood, which affects up to 5% of the population and is characterized by degraded vision in one eye not able to be corrected by spectacles and having no observable lesion in the eye itself. Its cause is therefore uncertain, yet indirect evidence suggests a neural one, very likely in the visual areas of the brain. Amblyopia is usually associated with either a strabismus (eye misalignment) or anisometropia (unequal refractive error). Investigation into its neural basis has involved two approaches: psychophysical research in humans with amblyopia¹⁻⁶ has shown marked contrast deficits for threshold stimuli, suggesting deficient contrast coding at and above threshold in amblyopia. Neurophysiological research on animals deprived in early life with either a surgically induced squint or an optically induced anisometropia has suggested that 'blur' may be the common etiological basis for these two amblyopias⁷. We have investigated here whether amblyopes exhibit contrast deficiencies for other than threshold stimuli. Our measurements show that the threshold deficits measured in amblyopia bear no simple relationship to what happens above threshold. There is no contrast coding abnormality in amblyopia in the high-contrast range typical of everyday vision. We also give suprathreshold evidence for anisometropic and strabismic amblyopia having different neural bases.

Repetitive spatial patterns with a sinusoidal luminance profile were generated independently on each side of a video display. Using a partition and a motor-driven sector disk (for alternate presentation of stimuli to each eye) an amblyope could be presented with the same pattern of independent contrast to each eye. Care was taken to ensure that all subjects were optically corrected. An experimental session began with the amblyope setting a contrast detection threshold for each eye at a particular spatial frequency. Gratings would then be set at any one of several contrasts above this threshold and presented to the amblyopic eye as the standard. The task was to adjust the contrast of the pattern seen by the good eye (variable) until it was perceptually equated to that seen through the amblyopic eye (standard). In some experiments this experimental protocol was reversed with the normal eye observing the standard contrast stimulus. Five settings were averaged for each contrast match using the method of adjustment.

In both figures the matching results have been plotted on logarithmic coordinates. If contrast matches between the eyes are set to equality then the results will fit along the diagonal line with a slope of 1. This is true for normal observers. Figure 1a and b which show contrast matching data for a representative strabismic amblyope and for a representative anisometropic amblyope are very different. For the strabismic amblyope (Fig. 1a), once the target contrast is just slightly above its detection threshold it is perceived correctly by the amblyopic eye. This result is independent of the bar size of the target (spatial frequency). However, for the anisometropic amblyope (Fig. 1b), contrast perception is degraded over a substantial suprathreshold range (0.5 log unit–1 log unit). This range is dependent on the spatial frequency of the pattern. These results suggest that even if the threshold losses for these two forms of amblyopia look similar, their suprathreshold perception is

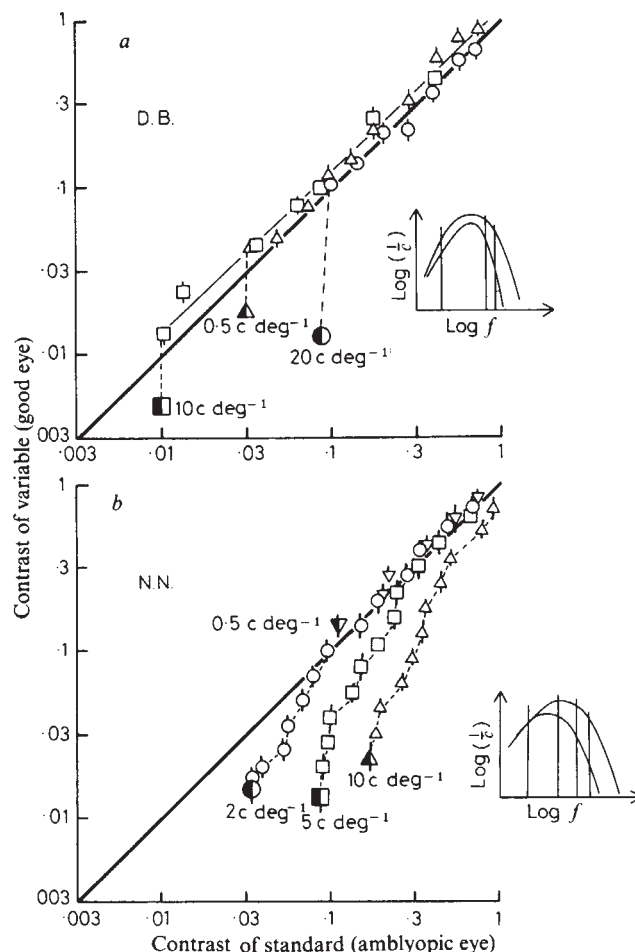


Fig. 1 Contrast matching results are displayed for three different spatial frequencies. The contrast of the standard grating shown to the amblyopic eye was matched by a variable grating of the same spatial frequency shown to the normal fellow eye. Each axis is logarithmic. Contrast thresholds are indicated by half-filled symbols. Inset is the form of the contrast threshold response for the normal and amblyopic eye. Strabismic (a) and anisometropic (b) amblyopia are compared. The gratings were displayed on a video screen for 500 ms. Field size of the stimulus was 10 deg, its mean luminance was 200 cd m⁻² and its colour was green (P 36 Phosphor).

dramatically different. This finding which has been confirmed for a group of strabismic and anisometropic amblyopes is summarized in Table 1 in terms of the contrast ratio between the eyes at threshold and at a contrast 3dB above threshold. An equivalent result is obtained when the contrast standard is shown to the normal eye. These results strongly suggest that strabismic and anisometropic amblyopes have different perceptual deficits.

These results also allow a better estimation of the type of neural dysfunction in human amblyopia. For example, strabismic amblyopia behaves as though the contrast range had been truncated to a different extent for each spatial frequency, thus maintaining normal suprathreshold contrast perception. Therefore, even though the contrast thresholds are raised in this form of amblyopia, this threshold disturbance has no suprathreshold relevance. It is as if a switch has been inserted somewhere in the amblyopic visual system to restrict the lower range of contrasts available to each set of frequency-specific neurones in the amblyopic visual system. To this extent the strabismic amblyope's system is well modelled by normal peripheral function. Figure 2a illustrates how normal contrast perception varies as a function of the position of a target in the peripheral field of vision for a normal subject. These results for normal peripheral viewing are very similar to that of the strabismic amblyope, supporting a more recent suggestion⁸ based solely on

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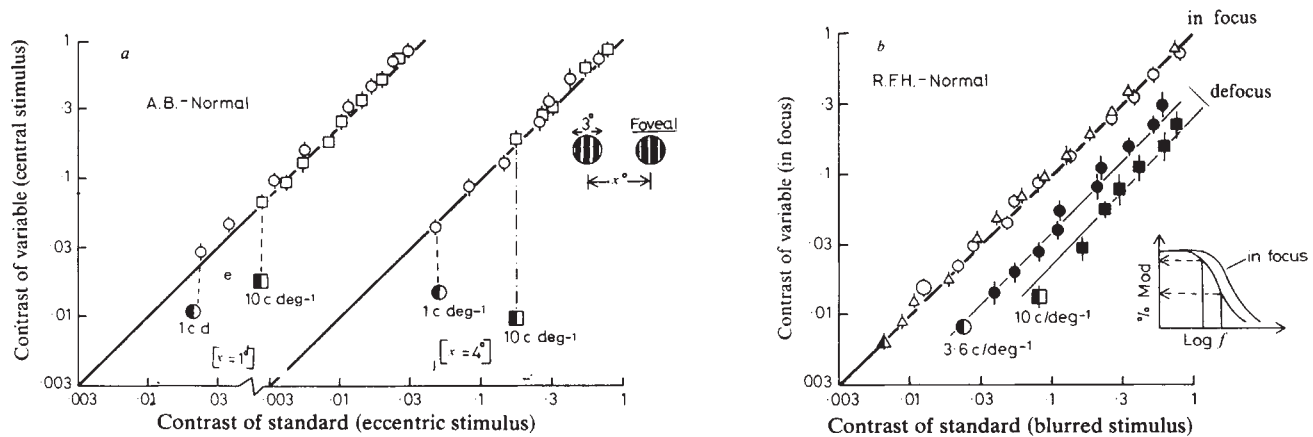


Fig. 2 Contrast matching results are displayed for a range of spatial frequencies. These results are plotted in an identical manner to those in Fig. 1. The contrast loss from two extents of eccentric viewing (1° and 4°) is displayed (a) as well as the contrast loss from optical blur (b). These should be compared with strabismic and anisometropic amblyopia (Fig. 1) respectively (see text).

threshold measurements that vision in strabismic amblyopia resembles that of the normal periphery.

The contrast matching abnormality is obviously different for the anisometropic amblyope (Fig. 1b); however the defective contrast perception does gradually diminish as the absolute contrast of the standard increases. Even for very fine targets (for example 10 c deg^{-1}) the defect at threshold is much larger than at high contrast levels (see also Table 1). It is interesting that the neural contrast deficit does not merely mimic the optical contrast loss due to the restricted (blurred) visual input present in early life. The contrast loss found with optical blur is displayed in Fig. 2b and this should be compared with the anisometropic result in Fig. 1b. Blur expected from a physical consideration should affect suprathreshold contrast perception as much as threshold perception (parallel displacement of the contrast matching line on log/log axes). Although optical blur (Fig. 2b) in early life may have originally produced the amblyopia seen in

Fig. 1b, it remains possible that substantial neural compensation may have occurred by the amblyopic visual system in the higher contrast range (compare Figs 1b and 2b in the upper range). This neural compensation may explain why adult amblyopes do not accept that the degraded vision from their amblyopic eye looks like a defocused version of what they see through their fellow normal eye. This compensation is best thought of as a gain change of about a factor of 2 for all spatial frequencies. It is as if an extra stage of amplification is present somewhere in the amblyopic visual system to boost the neural suprathreshold contrast signal in only the upper part of the range. A similar contrast gain change has been reported⁹ for the normal eye at high spatial frequencies and for some astigmatic subjects. These findings for contrast perception in subjects with abnormal vision are similar to those for loudness perception in subjects with peripheral damage to the auditory system. Within the auditory domain such a 'gain change' is referred to as 'recruitment'.

In conclusion our results strongly suggest that the contrast coding abnormalities in amblyopia are mostly limited to threshold conditions and also that the neural basis of anisometropic and strabismic amblyopia is different. They also highlight the nature of the suprathreshold contrast deficit in each condition. Although it has been shown that human amblyopes do not exhibit deficits in contrast perception in the mid- to high-contrast range, they do experience difficulty in identifying such stimuli. This is due to a somewhat independent anomaly in spatial perception. They see targets 'distorted'¹⁰, for example a simple black and white stripe pattern appears jumbled and twisted yet, as shown here, the individual components are perceived at their correct contrast. Clearly this spatial distortion is likely to be the main basis of amblyopia. The defining features reported here for the two main forms of human amblyopia should be amenable to neurophysiological assessment in animals which have been either strabismically or anisometropically deprived in the early months of life. Such assessment may indicate the relevance of animal models as well as the possible neural substrate for these human perceptual phenomena.

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Table 1 Comparison of threshold and suprathreshold abnormality (3 dB above threshold) for strabismic and anisometropic amblyopes

Subjects	Spatial frequency (c deg ⁻¹)	Threshold contrast ratio	Suprathreshold contrast ratio (a contrast 3dB above threshold)
(a) Strabismic amblyopes			
D.B.	0.5	0.5	1.25
	10.0	0.65	1.26
	20.0	0.16	1.01
S.C.	0.5	0.11	0.9
	1.0	0.1	0.95
	4.0	0.05	0.85
S.N.	0.25	0.16	1.12
	1.0	0.12	1.20
	3.0	0.05	1.12
F.B.	0.5	0.5	1.13
	1.0	0.61	0.97
	10.0	0.1	0.96
(b) Anisometropic amblyopes			
N.N.	2	0.40	0.37
	5	0.13	0.25
	10	0.14	0.16
A.H.	6	0.80	0.75
	10	0.45	0.50
	20	0.33	0.41
S.C.	6	0.40	0.50
	10	0.21	0.31
	15	0.15	0.30
A.C.	20	0.1	0.18
	1	0.3	0.5
	2	0.09	0.16
	3	0.017	0.1

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Basal lamina glycoproteins are produced by neuroblastoma cells

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Murine neuroblastoma cells have been widely used as a model system for neuronal cells as they can be induced to differentiate in culture by various stimuli, such as dibutyl cyclic AMP (dbcAMP)¹, prostaglandin², and serum starvation³. The cells respond with assembly of microtubules, leading to neurite outgrowth^{4,5}, with increased activity of neuronal-specific enzymes, tyrosine hydroxylase, choline acetyltransferase and acetylcholine-esterase^{6,7}, and synthesis of neurotransmitters⁸. The differentiated cells lose tumorigenicity⁹. Cell-to-substratum adhesion is evidently crucial for neurone extension *in vitro*¹⁰. Neurite outgrowth is induced by treatments that increase cell-to-substratum adhesion in some neuronal cell cultures^{11,12}. We have now identified the major high molecular weight proteins synthesized and secreted by murine C1300 neuroblastoma cells as fibronectin, laminin and type IV procollagen, of which the latter two were also found to be deposited in pericellular matrix form.

The mouse neuroblastoma cells (C1300, clone NB41A3; American Type Culture Collection, CCL 147) were cultured in Eagle's medium supplemented with 10% fetal calf serum and antibiotics. To induce morphological differentiation, the neuroblastoma cells were plated sparsely and cultured in the presence of 1 mM dbcAMP. The polypeptides secreted into the culture medium by metabolically labelled (³H-proline or ¹⁴C-glycine) neuroblastoma cells were precipitated with ammonium sulphate and analysed by SDS-polyacrylamide gel electrophoresis. The major polypeptide as seen on autofluorograms migrated with an apparent molecular weight (MW) of 230,000 (Fig. 1, lanes 2 and 3). In addition, labelled polypeptides were seen with MWs of 400,000 and 170,000. These polypeptides were disulphide-bonded to each other since similar analysis of unreduced samples revealed a major polypeptide band with a MW of 440,000 and larger material (Fig. 1, lane 1). The overall pattern of protein secretion did not appreciably vary over a wide range of labelling times and was not affected by the presence of 1 mM dbcAMP in the cultures (not shown).

Several major bands could be identified as either fibronectin or laminin polypeptides by precipitation from the neuroblastoma cell extracts and culture media with the respective antibodies (Fig. 1, lanes 4 and 5). The fibronectin chains showed an apparent MW of 230,000, while laminin polypeptides could be visualized as a closely spaced doublet with MW of 215,000 together with a higher molecular weight (400,000) component. The radiolabelled laminin polypeptides co-migrated with protein-stained¹³ components of authentic laminin purified from the mouse EHS sarcoma¹⁴ (not shown).

Initial analysis of medium proteins produced in the absence of ascorbate failed to identify collagenous protein. Since laminin has so far only been found together with collagen type IV both *in vivo*^{14,15} and *in vitro*^{16,17}, we also examined media of cell cultures labelled in the presence of sodium ascorbate and the cross-link inhibitor β -aminopropionitrile fumarate. Two new disulphide-linked polypeptides MWs 170,000 and 180,000 according to collagenous standards were now secreted and were sensitive to a highly purified bacterial collagenase (Fig. 1, compare lanes 2 and 3; 6 and 7). These two polypeptides comprised about 16%

of protein-bound ³H-proline-derived radioactivity and could be specifically precipitated with antibodies to type IV collagen (Fig. 1, lane 8). Antibodies to interstitial procollagens or collagens did not precipitate protein-bound radioactivity (Fig. 1, lane 9). The type IV procollagen polypeptides were particularly rich in the matrix material attached to the growth substratum (Fig. 2a) at the expense of fibronectin polypeptides, which were abundant in the medium (Fig. 2b). Indirect immunofluorescence studies using previously described procedures¹⁶ confirmed the presence of all three glycoproteins in the cells and deposition of laminin under the attached cells, as will be reported in detail elsewhere.

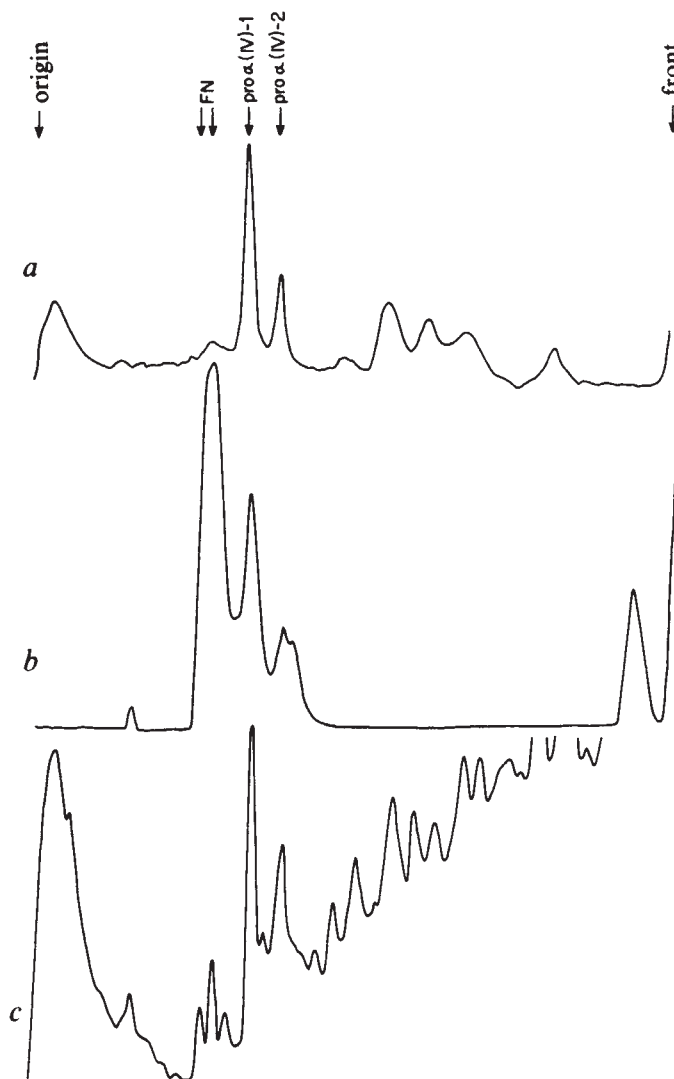


Fig. 1 Electrophoretic analysis³² of metabolically labelled proteins secreted into the medium by undifferentiated C-1300 neuroblastoma cells. Lane 1 medium, non-reduced; lanes 2, 3 and 7, medium reduced with mercaptoethanol. The medium in lanes 3, 6 and 7 was obtained in the presence of ascorbate ($30 \mu\text{g ml}^{-1}$) and β -aminopropionitrile fumarate ($30 \mu\text{g ml}^{-1}$); lane 6, medium after treatment with bacterial collagenase. Proteins were precipitated before analysis with ammonium sulphate (281 mg ml^{-1}) in the presence of gelatin carrier ($50 \mu\text{g ml}^{-1}$) and protease inhibitors³³. Lanes 4, 5 and 8, identification of proteins by immunoprecipitation¹⁶ with specific antibodies to laminin¹⁴ (lane 4), fibronectin¹⁸ (lane 5) and type IV collagen³⁴ (lane 8). Control precipitates in lane 9 were obtained either with anti-type II collagen or normal rabbit IgG. The polypeptides were visualized by fluorography³⁵; lanes 4 and 5 were exposed for 6 d, the other lanes for 3 d. Except for lane 1 all samples were analysed in reducing conditions. Globular proteins as indicated by their molecular weights (left side) and type I collagen and procollagen chains (right side) were used for calibration.

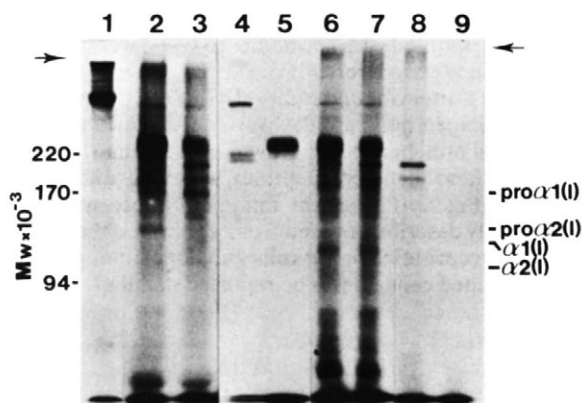


Fig. 2 Comparison of labelled proteins in the matrix (a), medium (b), and cell layer (c), obtained from undifferentiated C-1300 neuroblastoma cultures by densitometric scanning. Cell-free substrate-attached matrix was prepared using sequential extraction of cell layers with 0.5% sodium deoxycholate and hypotonic buffer in the presence of protease inhibitor³⁶. Cell layer contained both intracellular and matrix proteins solubilized directly into electrophoresis sample buffer. The positions of fibronectin (FN) and type IV procollagen pro- α 1 (IV) and pro- α 2 (IV) chains are indicated by arrows. Electrophoresis was carried out in reducing conditions.

No distinct deposits of fibronectin or type IV procollagen could be detected in cultures of either attaching or differentiating cells, but the latter was identified by electrophoresis (Fig. 2a). Essentially similar results were obtained with the NB42B clone⁵ of C1300 cells.

Several methods of determining the amounts of the major glycoproteins secreted by morphologically undifferentiated and differentiated C1300 cells gave no consistent differences between the two. This was the case when radioactivity in the high molecular weight peptide bands was plotted against cell number or amount of protein in the cell layer. Quantification of medium proteins by radioimmunoassay (RIA) using antibodies directed against human plasma fibronectin¹⁸ or the large, pepsin-resistant fragment of laminin¹⁹ gave similar values for both states, when corrected for the amount of cells in each culture. Nearly identical patterns for radioactive polypeptides were also observed in both cell layers, or isolated pericellular matrix material by electrophoresis followed by autofluorography or by RIA for pepsin-solubilized laminin.

These results show production of fibronectin, laminin and type IV collagen by two clones of a well-characterized line of murine neuroblastoma cells able to differentiate in culture. Cell-surface associated polypeptides of molecular weights similar to that of laminin and antigenically distinct from fibronectin have been detected both in N18 neuroblastoma²⁰ and in PC12 pheochromocytoma cell clones²¹. Furthermore, both nerve growth factor and dbcAMP have been found to induce alterations in protein secretion and substrate-attached material of PC12 cell structures²², whereas dbcAMP and subsequent differentiation had no effect on protein secretion in the present study.

The only collagenous protein secreted by neuroblastoma cells was of the basement membrane type. Its polypeptide pattern was very similar to that of type IV procollagen secreted by murine parietal yolk sac cells *in vivo*²³ and tumour cells in culture¹⁷. Note that secretion of collagen by neuroblastoma cells was detected only in ascorbate-treated cultures. The significance of type IV collagen for neuroblastoma cells remains unknown. Our data demonstrate that neuroblastoma cells in culture produce proteins characteristically found in a wide variety of basement membranes. It is possible that this synthesis pattern is an example of the so-called retrodifferentiation of tumour cells²⁴ and represent the primitive ectodermal origin of the (neuroblastoma) cells²⁵. Currently held views indicate that neurones lack adjacent basal laminae in the central nervous

system^{26,27} and that Schwann cells are responsible for matrix production in the peripheral nervous system^{28,29}. Intraneuronal synaptic clefts³⁰ and neuromuscular junctions³¹, however, contain matrix material. Thus it is also possible that basement membrane production by neuroblastoma cells, particularly in their differentiated state, reflects a similar *in vivo* activity.

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Corrigenda

In the letter 'Discovery of a very distant supernova' by M. R. S. Hawkins, *Nature* **286**, 467–469, in Table 1 the epoch of J4394 is 29.7.78. In Fig. 1 the point with the same ordinate as the cross is spurious and in Fig. 3 two of the points in the top left-hand corner are spurious. The sentence in line 44 on p. 468 beginning 'Note that...' should read 'In particular, the fit was made without the last two observations, which after galaxy subtraction were the most uncertain.' In line 3 on p. 469, for 'non-expanding Doppler' read 'non-Doppler'.

The following acknowledgement refers to the letter 'Renin-like effects of NGF evaluated using renin-antagonists' by D. B. Avrith *et al.*, *Nature* **285**, 248–250. 'We thank Professor Vernon, Department of Chemistry, University College London, for the gift of immunologically pure β -subunit of NGF'.

MATTERS ARISING

Geological objections to an extensional origin for the Buchan and Witchground Graben in the North Sea

CHRISTIE AND SCLATER'S refraction results¹ corroborate our inference from regional gravity² that the crust under the Buchan and Witchground Graben area of the North Sea is anomalously thin (see Fig. 1). However, their mechanism³ for the development of the grabens, invoking crustal stretching by a factor of ~ 2 during the late Jurassic to early Cretaceous, raises serious geological problems. (1) The structure of the inner Moray Firth at lower Mesozoic levels⁴ precludes both N-S extension of more than a few per cent, and significant later transcurrent movement on the Great Glen fault⁵. How can Christie and Sclater reduce their supposed N-S crustal extension of at least 60 km, along the Greenwich meridian, to zero, 200 km to the west? Our gravity modelling shows that if gross crustal extension occurred at all, it was in a NE-SW to E-W direction below the Viking and Central grabens, and not in a N-S direction across the Moray Firth. (2) The thickening of the Palaeozoic layer from near zero, at either end of their refraction profile, to about 4 km in the centre, implies up to 4 km of pre-late Permian erosion, and/or a Palaeozoic depositional basin. In contrast, their upper crustal layer seems to have an approximately constant thickness. The post-Zechstein, pre-stretching thickness of these two layers together was presumably, therefore, 5 km or less at the ends of the profile, but ~ 18 km in the centre. Whether or not erosion took place, it is clear that the crust was already highly anomalous by the mid-Permian so that the later, planar, 40-km deep Moho, depicted in Christie and Sclater's Fig. 4a as the initial (pre-stretching) crustal structure, is inconsistent with these implications from their own refraction results. (3) The major graben-boundary faults, probably active from the late Carboniferous (Stephanian) to the late Jurassic, are depicted (their Fig. 4a) as having had no effect on the Moho, whereas unobserved listric faults are postulated to have thinned the crust (their Fig. 4b) by a factor of ~ 2 shortly afterwards. Also not observed is the extreme rotation, of the order of 60° – 80° (ref. 6), of the Jurassic and older sediments required to produce such extension—actual tilts are only a few degrees. Well developed listric faults can now be recognized elsewhere on seismic reflection sections (for example, ref. 7), but can only account for upper crustal extension of $\sim 25\%$. (4) The effusion of a 3-km thick pile of mildly

under-saturated alkali olivine basalts in the centre of the refraction profile⁸ apparently pre-dates the initiation of the supposed stretching period by some 10 Myr, whereas rapid extension would probably result first in tholeiitic volcanism (particularly in the form of dyke swarms) before the more alkaline volcanism resulting from deeper partial melting. The only major pre-Tertiary tholeiitic dyke swarm in the region is considerably older, at ~ 290 Myr, preceding a major period of igneous activity in the Permian. In contrast, magmatism during the late Jurassic and early Cretaceous was trivial⁸.

In view of these problems we conclude that rapid extension of the scale required never occurred. Other mechanisms such as loading of an elastic or visco-elastic lithosphere^{2,9} can explain the last 120 Myr of North Sea subsidence just as readily as a thermal model, but without requiring such a catastrophic initiating mechanism.

M. J. Russell has suggested that the problem of the thin crust beneath the North Sea may be tackled by rejecting the assumption that the crust was originally of standard continental thickness and composition. The contiguity of the Scottish-Norwegian and North German-Polish Caledonides through the North Sea¹⁰, and

the implication, mainly from faunal provinciality, of three widely separated Lower Ordovician continental blocks¹¹, lead us to propose that the anomalous crust under the North Sea grabens may date back to the closing of a Lower Palaeozoic ocean. We therefore predict a small but significant difference in the seismic structure of the crust on either side of the grabens, as has been observed across the Iapetus suture¹² (Fig. 1). The later major tectonic and magmatic events of north-west Europe can all be ascribed, in principle, to the effects of nearby seafloor spreading in the early Permian¹³, early Cretaceous⁷ and the Tertiary, without resorting to an *ad hoc* stretching event.

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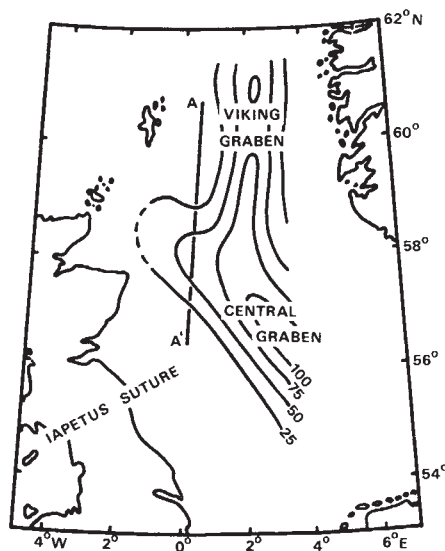


Fig. 1 Residual gravity anomaly (mGal) after removal of the effect of all Upper Palaeozoic and younger sediments (from ref. 2). AA' is refraction profile of ref. 1. Three-dimensional modelling predicts a Moho depth of 25 km below the centre of profile AA', on the lobe of overlap where the 200-km wide zone of thin crust below the Central Graben runs into the 130-km wide zone below the Viking Graben. Minimum Moho depth below the centre of each graben is 20 km, assuming a 'normal' Moho depth of 30 km and a density contrast 0.4 g cm^{-3} across the Moho.

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CHRISTIE AND SCLATER REPLY— We thank Smythe *et al.* for providing the opportunity to elaborate on the extensional model for basin formation¹. First, it cannot be denied that extension has taken place in the North Sea, and the strong temporal correlations between North Atlantic and North Sea activity^{2,3} enable us to refute the suggestion that the model is *ad hoc*.

As noted by us, evidence for the required extension must be found in the North Sea geological record for the model to be acceptable. Concerning the amount and type of extension, we would make the following points.

(1) Although the refraction line is a linear profile, this does not constrain the extension to be parallel to it or even one-dimensional and indeed the model describes areal extension⁴. Clearly, the geometry of the basin requires two-dimensional extension to have taken place in the vicinity of well 3 (ref. 4).

(2) Such extension, if azimuthally independent, would require linear extensions of 34 and 26% to produce the locally maximal values of b of 1.8 and 1.6 respectively⁴.

The amount of extension accommodated by listric faults is debatable. Although Montadert *et al.*⁵ suggest that listric-faulted blocks on the north Biscay margin, displaying tilts of 20° to 30°, allow some 15% extension, the same seismic reflection data have been interpreted by X. Le Pichon and J.-C. Sibuet (personal communication) to allow extension of 200% to 300%. Both Montadert *et al.* and Le Pichon and Sibuet cite extension as being the primary cause of crustal attenuation in this area, with post-rift subsidence the result of isostatic adjustment to cooling of the lithosphere.

We agree that magmatism during the Jurassic and early Cretaceous was trivial, justifying our assumption that crustal material was conserved. However, the apparent predating by basaltic effusions of the onset of extension by ~10 Myr seems more to support the placing of the stretching event in the Lower Jurassic than to refute the model.

The noted existence of a Permo-Triassic event in the North Sea may require an explanation of subsidence in terms of the superposition of two main stretching episodes. In this case, we would agree that the North Sea crustal thickness may already have been attenuated by the start of the Jurassic phase of activity, in which event, less Jurassic extension of even a one-dimensional type would be required to produce the seismic cross-section.

However, the good agreement between the seismic and gravity estimates of the depth to the Moho suggests that there is indeed crustal attenuation beneath the North Sea Basin, and 4 km of actual post mid-Cretaceous sedimentation is compelling evidence for something other than simple loading of an elastic lithosphere, which predicts a thickened crust⁶. A loading hypothesis would require the existence of a wide, 1,200-m deep depression at the time of the Aptian-Albian unconformity over a somehow previously thinned crust.

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Counting integral numbers of amino acid residues

THE method of “counting integral numbers of amino acid residues per polypeptide chain” described by Creighton¹ is not new either in concept or in execution. The idea of using varying ratios of iodoacetamide and iodoacetate to alkylate proteins and then to determine the number of alkylated cysteine residues by gel electrophoresis was first introduced by Feinstein² in 1966. In that original paper starch gel electrophoresis was used. Subsequently, Feinstein and Stott³ used isoelectric focusing to resolve the differently charged proteins. In both of those papers the ‘charge-shift’ technique was used to determine the number of free thiol groups on partially reduced proteins.

More recently Singer and I⁴ have used ‘charge-shift’ analysis of fully reduced protein to determine the cysteine content. We have therefore used the method in exactly the same manner as that described by Creighton. Extension of the charge-shift method to other amino acid residues is an obvious possibility. However, the data presented by Creighton relate only to the determination of cysteine residues.

I am most surprised that the previous publications on the charge-shift method should have escaped the attention of both Creighton and the *Nature* referees.

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CREIGHTON REPLIES—The papers cited by Williamson^{1–3} describing the ‘charge-shift’ method of determining cysteine residues, which has become the same in principle as the method I used⁴, were unfortunately unknown to me, the referee of my paper, all the colleagues with whom I discussed this matter, and the authors of all review articles and monographs on the subject of amino acid analysis that I could find. A thorough search through the index of *Amino Acid, Peptide and Protein Abstracts* for papers on amino acid analysis also failed to uncover these papers. This ignorance of the development of Feinstein’s original work² can be attributed to its publication only as a small part of papers dealing with the biosynthesis of immunoglobulins published in an immunological journal^{2,3}, with no mention of the method in either the titles or abstracts. This is an instructive example of how care must be taken if the attention of the appropriate audience is to be drawn to published work.

Having now studied these papers in detail, I note that the validity of the

charge-shift method was not established at that stage, either by confirming the results with an independent, established method or by demonstrating that it gave the correct results with a well characterized protein. It was also used inappropriately with a protein that was electrophoretically heterogeneous so that the results were ambiguous³. Demonstrating the validity of this method is especially important because it depends critically on the completeness and the specificity of the reaction of cysteine thiol groups with iodoacetate and iodoacetamide. I demonstrated this by obtaining single electrophoretic bands of unfolded proteins treated with the limiting cases of just iodoacetamide and just iodoacetate⁴. There was then no uncertainty about where to start and where to stop counting the bands generated by reaction with mixtures of the two reagents. The number of bands generated gave the correct number of cysteine residues for six well characterized proteins. When this procedure is used with a new protein, a well characterized protein should always be included as a control, to ensure the specificity and completeness of the reactions.

It is clear that this general method of determining integral numbers of specific amino acid residues is simple, easy, and can give unambiguous results when properly applied. The general approach should be useful for some amino acid residues other than cysteine; the observations of Anderson and Hickman⁵ using carbamylation and of M. Hollecker in this laboratory using succinic anhydride⁶ demonstrate that it may readily be extended to lysine residues.

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Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

BOOK REVIEWS

The genesis of experimental psychology

O. L. Zangwill

THIS book was compiled to mark the centenary last year of the establishment of the first laboratory in the world to be devoted exclusively to experimental psychology. Its founder was Wilhelm Wundt, a physiologist with a taste for philosophy, who had worked for many years at Heidelberg under the shadow of the great Helmholtz. After a somewhat undistinguished early career in physiology, Wundt was translated to Leipzig as a professor of philosophy, to which he was undoubtedly better suited, and at Leipzig he remained for the rest of his life.

In 1873, Wundt published the text on which his reputation has been principally based. In a later edition, this was translated into English by his devoted follower, E.B. Titchener, as *Principles of Physiological Psychology*. Of all Wundt's voluminous writings, this is, if not the best, certainly the best known. Wundt's own introductory statement to the original edition on "the task of physiological psychology" is one of the few extracts from his writings to appear in this volume which, however, also includes a fascinating review of the first German edition of the *Principles* by William James.

How did Wundt view experimental psychology? One may note first that whereas he saw physiological and experimental psychology as being virtually synonymous, he was in no doubt whatsoever that the latter warrants acceptance as a distinct and separate department of knowledge. This is well brought out in the second of the two excellent chapters by Kurt Danziger on Wundt's theoretical position. Arising from his exposition of Wundt's theory of human behaviour, Danziger directs special attention to the classical series of experiments on reaction times which were among the earliest fruits of his laboratory. Danziger treats the measurement of reaction times as in no sense a mere adjunct to physiology, as it had hitherto been viewed, but as a coherent manifestation of psychological research in its own right having direct relevance to the explanation of human behaviour.

As Wundt saw it, the most central of the issues underlying reaction time measurements was concerned with human volition. The distinction between "sensorial" and "muscular" reaction times, Wundt believed, had important theoretical implications for an understanding of volitional activity and its relationship to the evolution of higher-order choice and decision processes. Such issues, he considered, lie totally outside the

Wilhelm Wundt and the Making of a Scientific Psychology. Edited by R.W. Rieber. Pp.252. (Plenum: 1980.) £15.44, \$24.50.

province of physiology, which then even more than now was conceived to be limited to essentially reflex models of involuntary action. Indeed the prolonged neglect of Wundt's work in America was undoubtedly due, in part at least, to the protracted behaviourist hegemony and the reductionist philosophy which it embodied. Even in Germany, the belief that selective attention, so central to Wundtian thinking, lacked explanatory value, became a central tenet of Gestalt psychology and under its influence reaction times, too, ceased for many years to be of more than marginal interest.

Currently, behaviourism is clearly in decline, and cognitive and developmental preoccupations in psychology equally clearly in the ascendant. In consequence, the climate is once again more sympathetic to the Wundtian outlook and this book may itself result in a revival of interest in his work. True, concepts of information processing and control mechanisms have replaced the Wundtian concepts of volition, apperception and creative synthesis, the roots of which lie deep in the *Naturphilosophie* of mid-nineteenth-century Germany. As Danziger rightly points out, present-day psychology is concerned primarily with an understanding of human performance, governed by concepts of selective attention, information processing, and decision and choice. Such a psychology may, in some sense, widen rather than diminish the gap between psychology and the neurosciences, but it leaves unaffected Wundt's insistence on experimental

psychology being conceived as an autonomous discipline which stands in symbiotic relationship with physiology. Further, although Wundt regarded social psychology as an historical and cultural rather than as an experimentally based discipline, his emphasis on its pre-eminent importance for psychology in general will find a sympathetic response in many quarters today.

One particularly valuable contribution made by this book is the challenge it provides to the received ideas about Wundt and his work which stem largely from the writings of Titchener and his pupil, E.G. Boring, the celebrated historian of experimental psychology. This challenge emerges particularly clearly in Blumenthal's chapter on Wundt and early American psychology, though it is unfortunate that it was not thought appropriate to reprint his enlightening reappraisal of Wilhelm Wundt which appeared five years ago. Danziger has also some highly relevant things to say about Titchener's many misapprehensions of Wundt's philosophical position. Solomon Diamond contributes a long essay on Wundt's early life and activities as a physiologist, though his somewhat uninhibited personal criticism may be thought to limit its biographical value.

Although inevitably a hodge-podge, this book will be welcomed by many people who are genuinely interested in the origins and development of experimental psychology, even though some of them may still continue to believe, with William James, that psychology is less a science than the hope of a science. □

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HBV et al.

William S. Robinson

Hepatitis Viruses of Man. By A.J. Zuckerman and C.R. Howard. Pp.269. (Academic: 1980.) £16.80, \$39.

OCCASIONAL reviews of hepatitis viruses by experts are welcome because knowledge of these viruses has been advancing so rapidly over the past decade that it is sometimes difficult for those in the field, much less outsiders, to keep up and assimilate all of

the new findings. Such reviews are most helpful when they are able to provide new insights, bring some order where chaos reigns among conflicting data, evaluate new findings in the context of other relevant and better studied biological systems, or provide a bibliography which makes a body of research literature readily available to readers. The authors of this book are active investigators in the field and have a broad knowledge of recent work. They intended this book to provide "an account of the more important published advances and exciting developments in this field". The book does

provide a large amount of recent information about hepatitis viruses. It attempts to cover widely different aspects of such viruses, including their history, ultrastructure, antigenic structure, molecular structure, epidemiology, course of infection, pathology of associated diseases, immunology, laboratory testing, animal and cell culture infection, environmental control measures and management of infected patients, immunization and antiviral treatment. Most of the book by far is devoted to hepatitis B virus (HBV), less to hepatitis A virus and least to the newly recognized non A, non B hepatitis agents; coverage is thus in proportion to the amount of new information about each of the viruses.

Some aspects are more successfully covered than others. The first chapter, "The History of Viral Hepatitis", includes many important findings from the earliest recordings of hepatitis-like disease to recent breakthroughs that have propelled the field forward. This account is well documented with references. After this chapter the presentation changes. As each area is considered, recent findings are almost exclusively given, and older work is omitted or briefly summarized, often without referencing. Frequently statements are presented as fact without documentation. For example, a large part of the chapter "Pathology of the Liver in Acute Viral Hepatitis", is devoted to a near-textbook description of histological features of acute human viral hepatitis without documentation. By presenting only recent findings on epidemiology, only the surface of this subject is covered. Certain important and long-standing epidemiological concepts about these viruses are omitted such as the different epidemic and endemic patterns of infections, the different dynamics of the viruses in human populations and the

different factors which are involved in maintenance of each in populations, to name a few. In some places experimental results are given without crediting the investigators, for example on p.26. An experiment in which HBsAg was detected "in 18 out of 24 saliva specimens and in 10 out of 10 samples of semen" is presented without a reference. Similar statements are found throughout the book. This failure to document the source of some recent findings that are given in detail in the book and the almost complete omission of older findings and reference to literature (that before 1975) greatly limit the value of this book as a reference source for investigators in the field, teachers and students. The authors suggest that the earlier literature is fully cited in a previous book by one of them. This, of course, is only partially satisfactory since it requires simultaneous reading of both books to obtain the whole picture and it leaves this book as only a partial account.

The information in the book is usually presented as a sequence of descriptions of the findings of individual studies. Little attention is given to comparison of findings of different investigators, offering explanations of differences in findings, suggesting new approaches which might resolve differences, or organizing and analysing complex subjects. For example, e antigen is such a complex subject and a number of apparently conflicting findings have been reported. Claims suggesting that e antigen is an immunoglobulin, related to lactic dehydrogenase, or that it may represent a virion component, have been published. These different findings are not critically analysed nor is a judgement as to the merit of each offered. No approaches are suggested for resolving the differences. Conflicting data concerning the relationship of e antigen to liver disease are similarly handled.

The chapter entitled "The Nature of Hepatitis B Virus" is similarly a descriptive account of experimental findings about the virion. The reader is given little feeling that HBV is a virus in the modern sense with specific genes whose functions are involved in viral structure, infection and replication. Although few details of virus replication at the molecular level are available for HBV, there is evidence for the identity of some viral gene products, such as HBsAg polypeptides, and evidence for locations of viral antigen synthesis and the structure of viral DNA forms in cells.

Finally it is disappointing that the structural, molecular and biological features of hepatitis B that distinguish it from viruses of other groups were not especially pointed out in order to provide a perspective of this interesting and unique virus. In this regard, the recent finding of a similar virus in woodchucks was not included. The woodchuck virus is important not only because it indicates that HBV may be only one member of a new group of viruses, but because its association with hepatoma in infected animals offers one of the most compelling arguments for an important relationship of viruses with properties like those of HBV to primary liver cancer, an issue with HBV in man.

Thus, although this book provides much recent and important information about several viruses which are of great biological and clinical interest, the choice of material and its presentation limit the book's value as an introduction to these viruses and as a reference work for those already familiar with the field. □

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In memoriam: the English chalklands

Kenneth Mellanby

Ecology of the English Chalk. By C.J. Smith. Pp. 573. (Academic: 1980.) £23.80, \$55.

As a schoolboy I thought of the chalklands of England as a crocodile. A geological map hung on the wall of one of my classrooms, and its most prominent feature appeared to me to resemble a light-blue reptile with the North and South Downs as the upper and lower jaws, the body turning round so that the tail ended up in Norfolk and Lincolnshire. Living in Scotland and the north of England, I never visited my crocodile

until, as an undergraduate at Cambridge, I was taken by the botany department to study chalk grassland near Royston. I still remember my first visit, in spring, when the Pasque flower was at its best. This and many subsequent visits to Royston Heath, Salisbury Plain and other chalk grasslands continued to impress me with both the beauty and the scientific interest of these areas. Even when I dropped botany for entomology, the butterflies and other insects sustained my fascination.

For over 100 years naturalists have shared my enthusiasm, for even a cursory glance through the literature shows that more work has been done on chalk grassland than on any other plant and animal habitat. Dr C.J. Smith has performed a notable service in bringing the results of this careful study into one book. He lists well over 700 references, and he is clearly familiar with the information contained in them all.

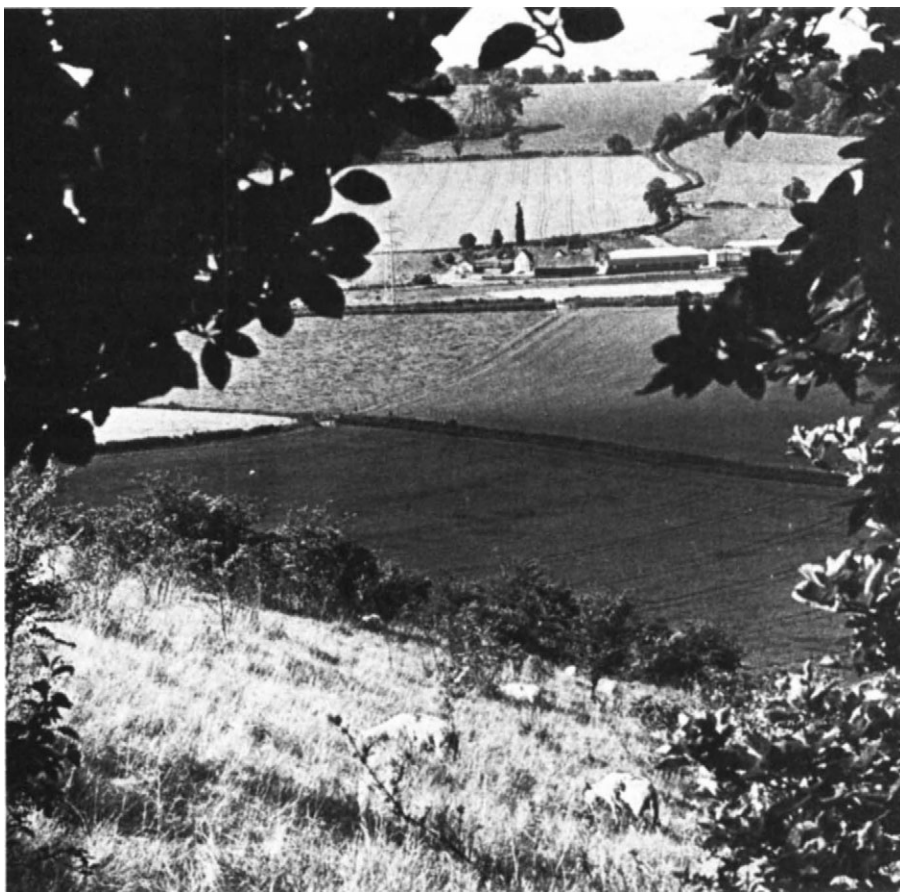
We are given a description of the chalk's geology and the history of the land use; there are details concerning the plants and animals and their interactions. The result cannot be called light reading: it is far too concentrated for that. But anyone interested in natural history who has visited the chalk will find something of interest, and something to stimulate him to further observation. Serious students will all acknowledge their indebtedness to Dr Smith's singleness of purpose, and this volume will be accepted for many years as the final authority on its subject.

The book makes it clear that the areas of chalk grassland which are of particular interest have all been profoundly affected by human activity. The land was first cleared of trees by our distant ancestors, mainly to grow arable crops. After a few years of declining fertility it was allowed to go down to grass, which was grazed by

stock — cattle and sheep. The best turf has existed for centuries. This species-rich grass only persists when it is regularly grazed, otherwise rank growth crowds out the most prized plants, and shrubs develop in the first stage leading to the forest climax. The scrub and woodland have their own interest and beauty, but it is the grass with all its flowers which has the greatest appeal. The sad thing is that it is this old grass which is so rapidly disappearing from England. It remains on only about 3% of the area covered 200 years ago, mainly as small fragmented patches on slopes too steep to cultivate. It is being replaced by fertile arable fields and improved, productive but species-poor grass. The modern farmer obtains many times the yield of former days — in doing so he improves the soil in structure and fertility, but he totally and irreversibly destroys the area from the point of view of nature conservation.

I am less optimistic than Dr Smith about the future of the fauna and flora of the chalk. Some unimproved grass will no doubt remain in nature reserves but, with the rise in land prices, economic pressures will probably affect the rest. So I welcome this book as a memorial to those who have worked on the chalk, and a record of one of the glories which is so surely departing from the English countryside. □

Kenneth Mellanby was director of Monks Wood Experimental Station, under the British Nature Conservancy, from 1961 to 1974.



Buttlers Hangings, a Naturalists' Trust Reserve, near West Wycombe in Buckinghamshire. The land here is too steep to cultivate, and represents the last remnant of chalk grassland and scrub between the woodlands of the plateau and arable land of the valley.

A milestone for the geologists of New Zealand

A. Ewart

The Geology of New Zealand. Chief editor R.P. Suggate. (Government Printing Office: Wellington, 1978/79.) Two-volume set NZ\$92.50 + postage.

IN 1950 the late R. W. Willett, then Director of the New Zealand Geological Survey, instigated an ambitious 1:250,000 mapping programme of New Zealand. Completed in 1968, the programme provided both stimulus and unity to geological research in New Zealand.

Only now, 15 years after the huge task of putting the results into print was started, have the efforts of geologists and editors culminated in the appearance of these two imposing volumes. Based on the contributions of 38 specialist authors, professionally produced and lavishly illustrated, the books represent the compilation and synthesis of a vast amount of data.

The organization of the text emphasizes the three distinctive tectonic phases through which New Zealand has evolved: the Tuhua Orogeny (mid to late Palaeozoic), the Rangitata Orogeny (mid to late Mesozoic) and the Kaikoura Orogeny (Miocene to the present day). The opening chapter provides the framework for the main text with sections on aspects of basic geology, and the following nine systematically treat, in great detail, the geology of New Zealand and its outlying islands, starting with the oldest sequences.

Six of the contributions lay down a descriptive basis. Each is concerned with a specific event and follows through the geological development of the region from the late Precambrian–Devonian to the Quaternary. Thoughtful editorial work is evident in that the format is consistent throughout: in each case an introduction leads on to consideration of the palaeontology, stratigraphy and petrology. Most notable is the profusion of illustrative material — maps, tables, correlation charts, photographs and line drawings litter the text. Interspersed between the descriptive chapters are three devoted to interpretation and synthesis of data on the three main orogenic phases.

The treatise concludes with a

consideration of palaeogeographical and palaeoenvironmental evolution, from the early Palaeozoic to Recent, as deduced primarily from the fossil record. This last chapter emphasizes the affinities and palaeogeographical links of the faunas and floras, and attempts to identify the gross climatic patterns and changes through the fossil record and their timing.

Shortcomings of the work are predictable, indeed inevitable, given the extended period of production and the number of authors involved. The contributions vary somewhat in style, objectivity and depth of treatment. In certain chapters overlap of topics is evident, and the material is not always internally consistent (for example the discussions of metamorphism in Chapters 4 and 5). Because the main text was prepared in the early 1970s the interpretations are made largely within the context of the New Zealand region alone, with only sporadic attempts at synthesis in terms of modern concepts of plate tectonics. This problem was recognized by the editors, however, and at the end of Vol. 2 they have provided a supplement to each chapter which consists of a brief review of developments up to about 1976, together with relevant references.

The Geology of New Zealand does not provide an easy introduction to the region and it will undoubtedly be used mainly for reference. However, publication of the volumes is certainly a milestone for geological research in New Zealand. They will provide a firm foundation for future research and should also be of great value

to all geologists concerned with the south-west Pacific, indeed to all those whose special interest is Mesozoic and Cenozoic geology. The chapters dealing with the history of these relatively younger rock sequences indicate how much New Zealand has to offer in such studies.

In all, this is an outstanding work. It is a

tribute not only to the editors and authors, but also to the strength and enthusiasm of the New Zealand Geological Survey. □

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Chemistry of steroids and peptides

J. S. Morley

Steroids and Peptides: Selected Chemical Aspects for Biology, Biochemistry, and Medicine. By J.B. Dence. Pp. 418. (Wiley-Interscience: 1980.) £20.85, \$47.50.

THE commendable general purpose of the author is to present the background of steroid and peptide chemistry to research workers in biology, biochemistry and medicine. Of the various classes of naturally occurring compounds, steroids and peptides are arbitrarily chosen for discussion (future volumes may deal with carbohydrates, lipids and nucleotides). They are discussed separately in introductory chapters, and in chapters on synthesis and chemical properties.

The opening articles will be the most

useful in that they deal with matters (occurrence, structure, nomenclature, physical properties and physiologically active species) of general interest to workers in other disciplines. The introduction to steroids is particularly well written. Even a modest background in chemistry should suffice to understand the clear accounts of seemingly complex topics — conformation, configuration, sequence rules, IR and UV spectroscopy, Woodward-Fieser rules and chirality, for example. Peptides are treated less well, and one is left with the impression that the author's experience of the field is drawn mainly from reading; perhaps this explains the often strange choice of examples. Nevertheless, basic information is well presented, and there is a useful summary of nomenclature rules (blatantly violated in later parts of the book!)

The chapter on steroid synthesis contains useful summaries of biosynthesis and steroid conjugates, but that on peptide synthesis may mislead rather than inform. There is, for example, no mention of protecting groups for C-terminal carboxy, and commonly used techniques are inadequately covered because of over-emphasis on newer, as yet unproven

methodology. It is unfortunate that coupling methods are illustrated by reference to an unsatisfactory synthesis of the L,D,L-isomer of thyroliberin; the methods involved, in contrast to those used in a later synthesis, lead to a partly racemic product. If a reader ever "should find himself in a situation where he must synthesise a peptide" he would be well advised to refer to one of the many specialist reviews of peptide synthesis rather than to this chapter.

The discussion of chemical properties cover oxidation, reduction, substitution reactions, derivatization and microbial transformations (steroids), and modifications at the N- and C-terminus and side-chain groups (peptides).

Returning to the aim of the book, the author has, I believe, wrongly assessed the needs of non-chemical research workers. If he had devoted more space to the topics discussed in the introductory chapters, and omitted or curtailed coverage of chemical synthesis, his book could have been more strongly recommended. □

J. S. Morley is Head of Peptide Research at the Pharmaceuticals Division of ICI Limited, Alderley Park, Cheshire.

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C. M. Yonge

British Crabs. By R.W. Ingle. Pp.222 (British Museum (Natural History)/Oxford University Press: 1980.) £16.

WHEN the decapod crustaceans learnt to tuck an increasingly reduced abdomen under a laterally expanding cephalothorax, the crabs had arrived. The well-enclosed gills permitted exploitation of intertidal and, in warmer lands, of terrestrial environments. The lateral scuttling movement proved ideal for seeking widely dispersed food and for insinuation within protective shelter.

Thomas Pennant separated these "crustaceous animals" from the insects where Linnaeus had placed them, and the fourth volume of his *British Zoology* (1777) contains figures of our commoner species. These include the male and female of the Long-clawed Crab, *Corystes cassivelaunus* (Pennant), here reproduced

to form a highly suitable frontispiece to a book representing the culmination of later studies initiated by William Elford Leach in his *Malacostraca Podophthalmata Britanniae* (1815-1820; completed by C. B. Sowerby in 1875).

Although less numerous here than in the tropics, there are 67 British crabs. Their long-needed modern description has now been admirably supplied in this authoritative book by Dr R. W. Ingle of the British Museum (Natural History). Initial accounts of early work on geographical and bathymetric distribution, and descriptions of external anatomy illustrated with accomplished line drawings, precede full systematic and descriptive information about all species. This is followed by 34 plates carrying double that number of extremely clear photographs. An exhaustive reference list completes a volume which will be welcomed by all marine biologists. It is a credit alike to author and to publisher, and a notable addition to works on carcinology. □

Sir Maurice Yonge is an Honorary Fellow in Zoology at the University of Edinburgh.